PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

TAMURA, Iwao
9-22, Terauchi 1-chome
Toyonaka-shi, Osaka 561-0872
JAPON

Date of mailing (day/month/year) 22 December 2000 (22.12.00)	
Applicant's or agent's file reference PTHJ-10008	**IMPORTANT NOTIFICATION**
International application No. PCT/JP00/01764	**International filing date** (day/month/year) 23 March 2000 (23.03.00)

1. The following indications appeared on record concerning:

☒ the applicant ☒ the inventor ☐ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
MIZUNO, Denichi 21-20, Okamoto 1-chome Kamakura-shi, Kanagawa 247-0072 Japan (applicant and inventor for all designated States)	JP	JP
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address	State of Nationality	State of Residence
MIZUNO, Denichi 21-20, Okamoto 1-chome Kamakura-shi, Kanagawa 247-0072 Japan (inventor for all designated States)		
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the International Preliminary Examining Authority
☒ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Shinji IGARASHI Telephone No.: (41-22) 338.83.38

M

09/700713

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

TAMURA, Iwao
9-22, Terauchi 1-chome
Toyonaka-shi, Osaka 561-0872
JAPON

RECEIVED
FEB 14 2001
TECH CENTER 1600/2900

Date of mailing (day/month/year) 22 December 2000 (22.12.00)	
Applicant's or agent's file reference PTHJ-10008	**IMPORTANT NOTIFICATION**
International application No. PCT/JP00/01764	**International filing date** (day/month/year) 23 March 2000 (23.03.00)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
MIZUNO, Denichi 21-20, Okamoto 1-chome Kamakura-shi, Kanagawa 247-0072 Japan (applicant and inventor for all designated States)	JP	JP
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Name and Address	State of Nationality	State of Residence
MIZUNO, Denichi 21-20, Okamoto 1-chome Kamakura-shi, Kanagawa 247-0072 Japan (inventor for all designated States)		
	Telephone No.	
	Facsimile No.	
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4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
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[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Shinji IGARASHI
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/01764

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A23K 1/16, 1/18, A61K 31/739, 31/00, 38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] A23K 1/16, 1/18, A61K 31/739, 31/00, 38/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS, JOIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, 9623002, A1 (Mizuno D.), 01 August, 1996 (01.08.96) & JP, 8-198902, A	1-16
X Y	JP, 6-141849, A (Soma Genichiro), 24 May, 1994 (24.05.94) (Family: none)	1-8,11-16 9,10
X Y	EP, 472467, A3 (Soma G.), 17 March, 1993 (17.03.93) & CA, 2049533, A & CA, 2049548, A & JP, 4-99481, A & JP, 6-78756, A & US, 5281583, A & JP, 6-40973, A & JP, 6-90745, A & US, 5346891, A & US, 5494819, A	1-8,11-16 9,10
A	JP, 8-280332, A (National Federation of Agriculture Coop. Assoc.), 29 October, 1996 (29.10.96) (Family: none)	1-16
A	JP, 10-279486, A (Taiyo Kagaku Co., Ltd.), 20 October, 1998 (20.10.98) (Family: none)	1-16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 May, 2000 (16.05.00)	23 May, 2000 (23.05.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer

INTERNATIONAL SEARCH REPORT

International application No. PCT/JP00/01764

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, 592220, A3 (Eisai Co., Ltd.), 23 November, 1994 (23.11.94) & JP, 6-116157, A & JP, 6-327412, A & JP, 7-41427, A & JP, 6-116158, A & US, 5556624, A & US, 5556625, A & US, 5624671, A & US, 5628998, A	1-16
A	Yukinori Takahashi, *Youshoku*, Vol.34, No.10, pp.117-121 (1997)	1-16
A	Fulvio Salati et al., *Nippon Suisan Gakkaishi*, vol.53(2), pp.201-204 (1987)	1-16
A	Marilyn J. Odean et al., *Infection and Immunity*, vol.58(2), pp.427-432 (1990)	1-16
A	L.W.Clem et al., *Development and Comparative Immunology*, vol.9, p.803-809 (1985)	1-16

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:

TAMURA, Iwao
9-22, Terauchi 1-chome
Toyonaka-shi, Osaka 561-0872
JAPON

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

Date of mailing (day/month/year) 12 April 2000 (12.04.00)	**IMPORTANT NOTIFICATION**
Applicant's or agent's file reference PTHJ-10008	International application No. PCT/JP00/01764

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

TAIHO PHARMACEUTICAL COMPANY, LTD. (for all designated States except US)
SOMA, Genichiro et al (all designated States)

International filing date : 23 March 2000 (23.03.00)
Priority date(s) claimed : 26 March 1999 (26.03.99)
Date of receipt of the record copy by the International Bureau : 07 April 2000 (07.04.00)
List of designated Offices :

AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EE,ES,FI,GB, GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK, MN,MW,MX,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA, ZW

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [X] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the InternationalSearching Authority.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer: Shinji IGARASHI
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

TENT COOPERATION TR[illegible]Y

From the INTERNATIONAL BUREAU

PCT

To:

TAMURA, Iwao
9-22, Terauchi 1-chome
Toyonaka-shi, Osaka 561-0872
JAPON

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 24 May 2000 (24.05.00)	
Applicant's or agent's file reference PTHJ-10008	**IMPORTANT NOTIFICATION**
International application No. PCT/JP00/01764	International filing date (day/month/year) 23 March 2000 (23.03.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 26 March 1999 (26.03.99)
Applicant TAIHO PHARMACEUTICAL COMPANY, LTD. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
26 Marc 1999 (26.03.99)	11/84399	JP	19 May 2000 (19.05.00)

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer Carlos Naranjo Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
TAMURA, Iwao
9-22, Terauchi 1-chome
Toyonaka-shi, Osaka 561-0872
JAPON

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 05 October 2000 (05.10.00)		
Applicant's or agent's file reference PTHJ-10008	**IMPORTANT NOTICE**	
International application No. PCT/JP00/01764	**International filing date** (day/month/year) 23 March 2000 (23.03.00)	**Priority date** (day/month/year) 26 March 1999 (26.03.99)
Applicant TAIHO PHARMACEUTICAL COMPANY, LTD. et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
   NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   05 October 2000 (05.10.00) under No. WO 00/57719

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase,** he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

(19) Europäis he Patentamt
European Patent Office
Office uropéen des brevets

(11) Publicati n number.: 0 477 050 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number : 91402275.1

(22) Date of filing : 20.08.91

(51) Int. Cl.[5] : C12N 1/20, C12P 19/04, A61K 37/22, // (C12N1/20, C12R1:01)

The microorganism(s) has (have) been deposited with Fermentation Research Inst. Agency of Ind. Science on Technology under numbers FERM P-11664, FERM P-11665, FERM P-11666 and transferred to the intern. deposit under Budapest Treaty on August 12, '91 under no. FERM BP-3509, FERM BP-3510, FERM BP-3511.

(30) Priority : 20.08.90 JP 218599/90
20.11.90 JP 312932/90

(43) Date of publication of application : 25.03.92 Bulletin 92/13

(84) Designated Contracting States : AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) **LPS-producing bacteria, LPSs, and LPS-containing medicines and veterinary medicines.**

(57) Novel LPS having the following physical properties, novel bacilli providing those LPS, and novel immunity stimulators, analgesics, and antiwithdrawal agents, and novel veterinary immunity stimulators, analgesics, and antiwithdrawal agents containing LPS selected from the group consisting of those LPSs and mixtures thereof.

LPS1
Dominant molecular weight :
5,000 ± 1,000 as determined by SDS-PAGE method
Phosphorus number :
2 ± 1 / 5,000 (m.w.)
Hexosamine number :
9 ± 1 / 5,000 (m.w.)
KDO number :
2 ± 1 / 5,000 (m.w.)

LPS2
Dominant molecular weight :
6,500 ± 2,500 as determined by SDS-PAGE method
Phosphorus number :
1 to 2 / 5,000 (m.w.)
H xosamine number :
7 ± 1 / 5,000 (m.w.)
KDO number :
1 to 2 / 5,000 (m.w.)

LPS3
Dominant molecular weight :

EP 0 477 050 A2

6,500 ± 2,500 as d termined by SDS-PAGE method
Phosphorus number:
2 ± 1 / 5,000 (m.w.)
Hexosamine number:
5 ± 1 / 5,000 (m.w.)
KDO number:
2 ± 1 / 5,000 (m.w.)

Field of the invention

Th present inventi n relates to nov l LPS-producing bact ria, nov l LPSS, and LPS-containing medicines and v terinary medicines.

More particularly, it is concern d with thre nov l glucose-fermentativ gram-n gativ small bacilli which produce LPSs, novel LPSs provided by those bacteria, and novel immunity-stimulating agents, analgesics, antiwithdrawal agents, veterinary immunity-stimulating agents, veterinary analgesics and veterinary antiwithdrawal agents containg those LPSs.

Description of the prior art

Organisms have their own immunity to keep their internal conditions from being disturbed by exogenous or endogenous matter and to maintain their homeostasis. Thus, the lowering of immunity causes deterioration of health, occurrence of various diseases, stimulation of aging, etc. On the other hand, its activation leads to improvement of health, prevention against occurrence of various diseases, cure of various diseases and prevention of aging or the like.

For the above-mentioned, it has been desired to provide a substance capable of activating immunity. To date, PSK [another name: Krestin^R (trade name of Kureha Kagaku Co. in Japan and registered in Japan], Lentinan^R (trade name of Ajinomoto Co. in Japan and registered in Japan), Bestatin^R (trade name of Nihon Kayaku Co. in Japan and registered in Japan), Sonifilan^R (trade name of Kaken Seiyaku Co. in Japan and registered in Japan), OK-432 *[Cancer Chemotherapy Reports Part 1, vol. 58, No.1, p.10, 1972*; another name: Picibanil^R (trade name of Chugai Seiyaku Co. in Japan and registered in Japan], etc. are known to have such capability.

Analgesics are classified into two groups; narcotic and non-narcotic ones.

Narcotic analgesics are of course narcotics, and thus they are required to be administered with the greatest care. *("Clinical pains", pp. 70 - 74, 1981, Medical Friend Co. in Japan)*

On the other hand, generally the analgesic action of non-narcotic analgesics is characterized to be less than that of narcotic ones and to be nonhabit-forming. But, actually their prolonged use is reported to cause tolerance and/or dependence of the patients thereto, and thus they are considered to be used in the same manner as narcotic-ones from pharmacological viewpoints. (*"Clinical pains", p. 74, supra*)

It is generally well known that the so-called withdrawal symptom occurs when one is suddenly kept from taking alcohol, morphinic narcotics, nicotine, etc. to which he has become addicted. Also it is well known that addicts of them are hard to return to daily life, and the clinical use of narcotics is restricted because of withdrawal symptons.

To date, methadone, clonidine, dizocilpine, etc. are known as medicines for suppressing such withdrawal symptoms. Methadone is, however, reported to cause dependence to itself. (P. R. Dougherety, et al., "Neuropharmacology", 26, pp. 1595 - 1600, 1987) Clonidine is reported to suppress withdrawal body shake by intraperitoneal administration of 0.16 mg/kg. (*Stuart Fielding, et al. "The Journal of Pharmacology and Experimental Therapeutics", vol. 207, No. 7, pp. 899 - 905, 1978*) But, we, the inventors, have found that intravenous administration of clonidine fails to suppress jumping, a severer withdrawal symptom even at a dose of 0.1 - 10 mg/kg, and further causes convulsions at a dose of 10 mg/kg. Dizocilpine shows only an extremely small difference between its toxic and effective doses, and thus is not safe. (*Keith A., et al., "Science", 251, pp. 85 - 87, 1991*)

Of the prior art immunity stimulators, PSK, Lentinan^R, Bestatin^R and Sonifilan^R have no TNF productivity, and thus their immunity stimulation is poor.

Surely OK-432 is known to have TNF productivity, but a rather large quantity of it must be administered to produce a satisfactory quantity of TNF, thereby inevitably causing attack of fever or rigor, lowering of blood pressure, and reduction in the number of thrombocytes. Accordingly, OK-432 has a low therapeutic range. OK-432 has an additional drawback in that its production steps include culture of microorganisms, and extremely complicated procedures for its separation and purification to increase the production cost. Further, OK-432 fails to produce TNF through oral or subcutaneous administration which is very convenient for medication; therefore OK-432 must be administered by inconvenient means.

Here, the term "TNF" is the generic name for tumor necrosis factors produced by macrophage (*The Journal of Biol. Chem., <u>260</u>, pp. 2345-2354, 1985*), and the production quantity of TNF increases depending on the activity of macrophage. Macrophage is the generic name for large amoeba-like cells which belong to immunocomp tent cells, are present in most int rnal tissues of animals, and prey and digest particulat foreign matt r and wast cells in th body. Th term "th rap utic rang " is th ratio f the maximum tol rant dose of th host to the medicin to th minimum ffectiv d s of th medicin ; th larg r th ratio is, the better th medicine is.

As mentioned above, the prior art analgesics have drawbacks, and no satisfactory nes have not been provided y t. Particularly, analgesics which are effectiv against chronic pain, are highly safe, hav n sid effects, are cheap and are very conveni nt for medication hav been greatly expected to be dev loped. Also no satisfactory antiwithdrawal agents have not been provided yet.

The pres nt inv nti n is int nded to provid n v l immunity-stimulating ag nts, analgesic ag nts, antiwithdrawal agents, veterinary immunity-stimulating agents, veterinary analgesic agents and veterinary antiwithdrawal agents which are free from the drawbacks of the prior art.

An additional object of the present invention is to provide novel LPSs, active ingredients of those agents, which have excellent immunity-stimulating, analgesic and antiwithdrawal effects, show a high therapeutic range, and may be provided at a low cost and in a large amount and may be administered via any route of oral and intradermal administration and injection.

An additional object of the present invention is to provide novel bacteria which produce the novel LPSs.

Brief description of the drawings

Fig. 1 is a chart showing the patterns of the LPSs of the present invention on SDS-PAGE method.

Fig. 2 is a graph showing the analgesic effects of the LPSs of the present invention in comparison with E. coli LPS.

Fig. 3 is a graph showing the dose-dependent antiwithdrawal effects of the LPSs of the present invention in intravenous administration.

Fig. 4 is a graph showing the dose-dependent antiwithdrawal effects of the LPSs of the present invention in subcutaneous administration.

Fig. 5 is a graph showing the administration time-dependent withdrawal-preventive effects of the LPSs of the present invention.

Detailed description of the invention

<u>Bacteria-providing sources</u>

The three bacteria according to the present invention were isolated from all kinds of wheat investigated by the inventors of the present invention regardless of their places of production. Thus, those bacteria are supposed to be isolated from any kind of wheat produced in any place and its processed goods. The kinds and the places of production of the wheat flour from which the three bacteria mentioned above were confirmed to be isolated by the inventors of the present invention include the following:

Kinds of wheat flour	Places of production
Dark Northern Springs	U.S.A.
1 Canadian Wheat	Canada
Hard Red Winter Semi-hard	U.S.A.
Australian Standard Wheat	Australia
Horoshiri	Japan

<u>Isolation of LPSs</u>

The LPSs of the present invention may be isolated from the above bacteria by the hot phenol process described on page 83 of Westphal, et al., "*Methods in Carbohydrate Chemistry*", *vol. v, 1965, Academic press in New York*, followed by purification on an anion-exchange resin.

That is, the cells are suspended in distilled water which is then stirred with an equivolume of hot phenol. Next, the aqueous layer is recovered by centrifugation and then subjected to dialysis to remove off the phenol. The aqueous layer is concentrated by ultrafiltration to yield crude LPS fractions which are then purified conventionally, for example, by anion-exchange chromatography using mono Q-Sepharose and Q-Sepharose in FPLC system (all manufactured by Pharmacia Inc.), followed by desalting in a conventional manner.

Products of 96% or more purity are provided by the foregoing procedures.

<u>Physical properties of LPSs</u>

As xplain d in d tail in the examples giv n lat r, th three LPSs of th present inv ntion having a purity of 96% or more showed the following physical properties ("SDS-PAGE method will be d fined later in Example 1):

LPSI	
Dominant molecular weight:	5,000 ± 1,000 as determined by SDS-PAGE m thod
Ph sphorus number:	2 ± 1 / 5,000 (m.w.)
Hexosamine numb r:	9 ± 1 / 5,000 (m.w.)
KDO number:	2 ± 1 / 5,000 (m.w.)
LPS2	
Dominant molecular weight:	6,500 ± 2,500 as determined by SDS-PAGE method
Phosphorus number:	1 to 2 / 5,000 (m.w.)
Hexosamine number:	7 ± 1 / 5,000 (m.w.)
KDO number:	1 to 2 / 5,000 (m.w.)
LPS3	
Dominant molecular weight:	6,500 ± 2,500 as determined by SDS-PAGE method
Phosphorus number:	2 ± 1 / 5,000 (m.w.)
Hexosamine number:	5 ± 1 / 5,000 (m.w.)
KDO number:	2 ± 1 / 5,000 (m.w.)

Forms supplied

The LPSs of the present invention may be supplied as such or in forms concentrated to any desired degree. Further, they may be supplied as dry powders by any of the conventional manners including lyophilization and spray drying to improve stability. Any of these forms may be produced conventionally.

Determination of immunity stimulation

The immunity stimulation of the LPSs according to the present invention has been confirmed by endogenous TNF productivity.

Carswell et al. report that priming and triggering steps are necessary to produce endogenous TNF in the body of an animal; see *Proc. Natl. Acad. Sci. USA., 72, pp. 3666 - 3670, 1975*. Thereafter, many candidate chemicals were tried to stimulate the respective steps. The chemical used to start the priming step is a primer (endogenous TNF production stimulator), while that administered to start the triggering step is a trigger (endogenous TNF productive agent).

The TNF activity is determined, as follows, on the basis of the cytotoxicity to L929 cells (*Proci. Natl. Acad. Sci. U.S.A., 72, pp. 3666 - 3670, 1975*). L-929 cells are cultured in Eagles' Minimum Essential Medium (hereunder referred to only as MEM) with 5 % fetal calf serum (hereunder referred to only as FCS) added thereto until 100 $\mu\ell$ of the medium contains 8 x 10^4 cells, and then the cells are grown in a flat-bottomed plate having 96 wells.

The growth conditions are 37°C in the presence of 5 % CO_2, and under a humidity of 100 % for 2 hours, and the procedures may be the same as for the conventional cell culture. Then actinomycin D is added to the medium to a final concentration of 1 $\mu g/m\ell$, and the volume of the culture solution is adjusted to 150 $\mu\ell$. Immedrately thereafter 50 $\mu\ell$ of the sample diluted appropriately with MEM medium is added to the culture solution. Here, ED_{50} may be determined by adjusting the dilution appropriately. The L-929 cells having a final volume of 200 $\mu\ell$ are cultured for an additional 18 hours under the same conditions as described above.

In order to determine the cell necrosis activity, first the whole medium is removed followed by addition of a 1% methyl alcoholic solution containing 0.1 % crystal violet for fixation staining. Crystal violet stains all the eukaryotic cells, but the dead cells are removed off from the bottom of the flask only by washing after the staining; so the cell necrosis activity may be determined directly. The staining degree is measured on the basis of adsorption at OD_{590nm}, and is compared with that of a control to determine the cell necrosis activity. This activity is defined as follows.

The dilution of the sample which allows 50 % of the L-929 cells to survive (N) is determined. Rabbit TNS is used as the control, and its activity n (units/ml) is determined using 2.4 x 10^6 units/mg/mℓ of TNF-α. The dilution which provides ED_{50} of rabbit TNS is determined.

The activity of the sample (units/ml) is calculated by the equation: N/C x n.

Determination of analgesic effects

Th analgesic eff cts of th LPSs of th pres nt invention have be n confirmed by an xperim nt using animals according t th ac tic acid-writhing method described on pag 415 of "Inflammation and anti-inflammatory therapy" issu d in 1982 by Ishiyaku Shuppan Co. in Japan, one of the established methods f r the d ter-

minati n of the effects of n n-narcotic analgesics.

D termination f antiwithdrawal ffects

The antiwithdrawal effects of th LPSs according t th present inv ntion hav be n confirmed by th reduction in the frequency of jumping, the severest withdrawal symptom caused by the administration of naloxone to morphine-addictive mice. Naloxone is available from Endo Labs. Inc. in U.S.A., and is known to be a morphine antagonist; "*The Journal of Pharmacology and Experimental Therapeutics*", *vol. 207, No. 7, p. 901, supra.*

The LPSs according to the present invention may be used separately, and further may be used in admixture with each other or together with any other substance so far as the intended effects are not made less. In addition, they may be ingredients of immunity diagnosis reagents, veterinary immunity diagnostic reagents, quasi drugs defined in the Japanese Pharmacopoeia, cosmetics, food, drinks and feed.

Any of the above preparations including immunity stimulators may be produced conventionally. For example, in the conventional manner of preparing medicines or veterinary medicines, they may be supplied conventionally in the form of powders, granules, pills, tablets, troches, capsules, solutions, pastes, ointments, liniments, lotions, suppositories, injections, etc. Paticularly, many macrophages are present in the skin, so the LPSs of the present invention may be prepared as skin ointments in order to obtain better effects. For veterinary use, also the agents may be prepared in the form of feed additives, premix preparations, drinking water additives. Here, the "premix preparations" are such preparations as contain feed components beforehand so that they are easily mixed in the feed. The feed additives are preferred to be powders or granules. Any commercially available feed may be used to prepare the above -mentioned feed additives, premix preparations, etc. The feed may contain minerals, vitamins, amino acids and any other feed additives.

If desired, these preparations may contain excipients, preservatives, buffers, etc. conventionally to improve the shelf life, homogeneity, etc. In addition, the preparations may contain correctives to improve taste, odor, appearance, etc. The excipients include, for example, lactose, starch, etc. The preservatives include, for example, parahydroxybenzoic esters such as methyl, ethyl or propyl paraoxybenzoate, sodium dehydroacetate, phenols, methyl, ethyl or propylparabene, etc. The buffers include, for example, citric, acetic or phosphoric acid salts, etc.

Hereunder, the present invention will be explained in detail with reference to examples, preparations and experiments. The E.coli LPS used therein is one available from Difco Co. in U.S.A. under the code numer of 0128:B8

Example 1

1) In a 50 ml coning tube, there was charged 1.04 g of hard flour containing 1.09% of ash (1 Canadian wheat from Canada) followed by addition of 20mℓ of distilled water thereto to prepare a 50 mg/ml aqueous solution of wheat flour.

2) The solution was cultured in a water bath at 37°C while shaking, and 0.5 ml portions of the solutions were collected at 0, 1, 2, 3, 4, 6, 8, 10, 12, 20, 24 and 45 hours thereafter. 100 μl portions of the respective solutions diluted to 1 to 100,000 times were taken in standard agar culture media available from Nissui Seiyaku Co. in Japan and having the following composition to determine the number of living cells and to observe the colonies.

Standard agar culture media (Nissui Seiyaku's code No.: 05618)

Yeast extracts	2.5 g/l
Peptone	5.0 g/l
Glucose	1.0 g/l
Agar	15.0 g/l
pH	7.1±0.1

At the end of 8 and 10 hour culture, yellow to creamy opaque colony (colony 1), creamy opaque colony (colony 2) yellow translucent colony (colony 3), milk white opaque colony (colony 4), and white opaque small colony (colony 5), which were judged to be different from each other, were scattered on the respective standard agar culture having the same compositi n as the above, for subcultivati n. The gram staining and limulus activity of th bacteria in th col nies w re d t rmin d.

H re, th "limulus activity" means to be positiv to limulus test which is a method inv nted by Levin in 1968 for quantitativ d termination of endot xin using a n rseshoe crab haemocyte extract and a chromogenic sub-

strate. The limulus test is known as a method for the detection of LPSs, and may be carried out using, for exampl , a reagent s t comm rcially available from S i-Kagaku Kogyo Co. in Japan under the trad name of Toxi Col r system.

Of the abov colonies, the limulus activity of th colonies 4 and 5 (both being gram stain-positive) were xtremely low as compared with that of the col nie 1, 2 and 3 (all being gram stain-negativ), so th former colonies were taken aside. The morphological and biochemical characteristics of only the colonies 1, 2 and 3 were observed using the media available from Nissui Seiyaku Co. and ID tests EB-20 to show the following results:

Bacteria forming the colony 1 (ID number: 900814-1)

(The bacteria were depositted with Fermentation Research Institute Agency of Industrial Science and Technology on August 17, 1990 under the number of FERM P-11664 and transferred to the international deposit under BUDAPEST TREATY on August 12, 1991 under the number of FERM BP-3509.)

The bacteria are supposed to belong to the genus Serratia of the family Enterobacteriaceae in view of the following morphological and biochemical characteristics.

a) Morphological characteristics
1) Small rod
2) No Motility
3) Gram stain: -

b) Growth
1) Standard agar medium: a yellow to creamy round opaque colony is formed.
2) SS agar medium: A white translucent colony is formed.
[SS agar medium: Nissui Seiyaku's code No. 05031]

Broth	5.0 g/l
Bile acid salts	9.0 g/l
Peptone	7.5 g/l
Lactose	10.0 g/l
Sodium citrate	8.5 g/l
Sodium thiosulfate	5.5 g/l
Ferric citrate	1.0 g/l
Neutral red	0.025 g/l
Brilliant green	0.033 g/l
Agar	13.5 g/l

pH: 7.1± 0.1

3) TSI agar medium: No change is found on the slant, but the higher layer changes to yellow. Gas if produced.
[SS agar medium: Nissui Seiyaku's code No. 05031]

Broth	5.0 g/l
NaCl	5.0 g/l
Peptone	15.0 g/l
Lactose	10.0 g/l
Sucrose	10.0 g/l
Glucose	1.0 g/l
Ferric citrate	0.2 g/l
Sodium thiosulfate	0.2 g/l
Phenol red	0.02 g/l
Agar	15.0 g/l

pH: 7.6± 0.1

c) Physiological characteristics
1) Voges-Proskauer reaction: +
2) Indole production: -
3) Hydrogen sulfide production:-
4) Utilization of citrate: +
5) Urease: -
6) Oxidase: -
7) O-F test: +
d) Utilization of carbon sources
1) Lactose: +
2) Adonitol: -
3) Rhamnose: +
4) Mannitol: +
5) Esculin: +
6) Inositol: -
7) Sorbitol: +
8) Arabinose: +
9) Raffinose: +
10) Sucrose: +
e) Others
1) Lysin decarboxylase: -
2) Utilization of malonate: -
3) Arginine dihydroxylase: -
4) Phenylalanine deaminase: -
5) Ornithine decarboxylase: -

Bacteria forming the colony 2 (ID number: 900814-2)

(The bacteria were depositted with fermentation Research Institute Agency of Industrial Science and Technology on August 17, 1990 under the number of FERM P-11665 and transferred to the international deposit under BUDAPEST TREATY on August 12, 1991 under the number of FERM BP-3510.)

The bacteria are supposed to belong to the g nus Enterobacter of th family Ent robacteriaceae in view of th following morphological and bioch mical characteristics.

a) Morphological characteristics
1) Small rod

2) No Motility
3) Gram stain: -

b) Growth
1) Standard agar m dium: a creamy opaqu col ny is f rmed.
2) SS agar medium: A red opaqu col ny is f rmed.
3) TSI agar medium: No change is found on the slant, but the higher layer changes to yellow. Gas if produced.

c) Physiological characteristics
1) Voges-Proskauer reaction: + ,
2) Indole production: -
3) Hydrogen sulfide production: -
4) Utilization of citrate: +
5) Urease: -
6) Oxidase: -
7) O-f test: +

d) Utilization of carbon sources
1) Lactose:,+
2) Adonitol: -
3) Rhammnose: +
4) Mannitol: +
5) Esculin: +
6) Inositol: -
7) Sorbitol: +
8) Arabinose: +
9) Raffinose: +
10) Sucrose: +

e) Others
1) Lysin decarboxylase: -
2) Utilization of malonate: +
3) Arginine dihydroxylase: +
4) Phenylalanine deaninase: -
5) Omfthine decarboxylase: +

Bacteria forming the colony 3 (ID number: 900814-S)

(The bacteria were depositted with Fermentation Research Institute Agency of Industrial Science and Technology on August 17, 1990 under the number of FERM P-11666 and transferred to the international deposit under BUDAPEST TREATY on August 12, 1991 under the number of FERM BP-3511.)

The bacteria are supposed to belong to the genus Pantoea of the family Enterobacteriaceae in view of the following morphological and biochemical characteristics.

a) Morphological characteristics
1) Small rod
2) No Motility
3) Gram stain: -

b) Growth
1) Standard agar mediun: A yellow round translucent colony is formed.
2) SS agar medium: No colony is formed.
3) TSI agar medium: No change is found on the slant, but the higher layer changes to yellow. Gas in not produced.

c) Physiological characteristics
1) Voges-Proskauer reaction: +
2) Indole production: -
3) Hydrogen sulfide production: -
4) Utilization of citrate: +
5) Ureas : -
6) Oxidase: -
7) O-F test: +

d) Utilization of carbon s urces

1) Lactose: +
2) Ad nitol: -
3) Rhamnose: +
4) Mannitol: +
5) Esculin: +
6) Inositol: -
7) Sorbitol: +
8) Arabinose: +
9) Raffinose: -
10) Sucrose: +

e) Others

1) Lysin decarboxylase: -
2) Utilization of malonate: +
3) Arginine dihydroxylase: -
4) Phenylalanine deaminase: -
5) Ornithine decarboxylase: -

4) The colonies 1, 2 and 3 were transferred to 1 liter L-broth medium, respectively, and the media were shaken at 37°C over night, and then subjected to centrifugation at 5,000 G, 4°C for 20 minutes to collect the cells. The L-broth was prepared by dissolving 10 g of polypeptone (Difco Co.), 5 g of yeast extracts (Difco Co.) and special grade NaCl (Wako-Jun-Yaku Co. in Japan) in distilled water, adjusting the pH of the solution to 7.5 with NaOH followed by autoclaving, and then adding a 400-fold dilent of a 40% solution of special grade glucose (Wako-Jun-Yaku Co.) to the solution.

5) The cells of the respective colonies were suspended in 50 ml of distilled water, and 50 ml of a 90 % hot phenol was added to the suspension followed by stirring at 65 - 70°C for 20 minutes. After being cooled, the mixture was subject to centrifugation at 10,000 G, 4°C for 20 minutes to recover the aqueous layer. The phenol layer was treated additional two times in the same manner as the above. The combined three aqueous layers were subjected to dialysis overnight to remove the phenol. The inner solution was subjected to ultrafiltration using UK-20 (Advantec Toyo Co.) for concentration by cutting off molecular weight 200,000; N_2 pressure: 2 atms.

6) The concentrated sample was subjected to anion-exchange chromatography using Q-Sepharose Fast Flow (Pharmacia Co.). That is, the sample was applied to the column using a buffer solution containing 10 mM Tris-HCl (pH 7.5) and 10 mM of NaCl, and then the limulus active fractions were eluted with 400 mM NaCl / 10mM Tris-HCl (pH 7.5). The eluate was subjected to ultrafiltration unde the same conditions as the above for desalting and concentration to produce 96% or more pure LPS. The nucleic acid was eluted with 1 M NaCl / 10 mM Tris-HCl (pH 7.5).

The results of the respective cells are shown in Tables 1 - 3. Here, the LPS content is in terms of E. coli LPS. The sugar content was determined according to the phenol - sulfuric acid method (M. Dubis et al., "Analytical Chemistry", vol. 28, p. 350, 1956), while the protein content was determined by the Lowry method (O.H. Lowry et al., "Journal of Biological Chemistry), vol. 193, p. 65, 1951. The nucleic acid content was determined on the basis of the measurements of OD at 260 nm (1 OD= 50 μg), and the purity (%) was calculated by the equation:

$$\text{Purity} = \frac{\text{Dried yield} - (\text{Protein yield} + \text{nucleic acid yield})}{\text{dried Yield}} \times 100$$

Table 1: 900814-1

Total dried yield (mg)	6.8
LPS (mg)	19.8
Sugar (mg)	3.1
Protein (μg)	86
Nucleic acid (μg)	<161
Purity (%)	96<

Table 2: 900814-2

Total dried yield (mg)	10.4
LPS (mg)	75.6
Sugar (mg)	2.5
Protein (μg)	64
Nucleic acid (μg)	<108
Purity (%)	98<

Table 3: 900814-3

Total dried yield (mg)	19.2
LPS (mg)	103.6
Sugar (mg)	7.6
Protein (μg)	73
Nucleic acid (μg)	<137
Purity (%)	99<

6) Molecular weight

The LPSs resulting from the respective cells were dissolved in distilled water, respectively to prepare solutions containing 2 mg/ml of LPSs. The 10 μl portions of the solutions were placed in 1.5 ml plastic tubes. To the respective portions there was added 10 μl of an SDS treatment solution prepared by mixing 10 μl of 10 % (w/v) of SDS, 45 μl of 5 % β-mercaptoethanol, 90 μl of a CBB coloring matter solution, 112.5 μl of 0.5 M Tris-HCl (pH 6.8) and 22.5 μl of distilled water. The resulting mixture was mixed well and then immerssed in boiling water for 5 minutes, and immediately thereafter the mixture was quenched in ice water.

10 ml of 10 % (w/v) SDS, 17.9 g of tricine and 3.03 g of Tris were dissolved in 1 liter of distilled water to prepare a buffer solution for electrophoresis which was then placed in Slab-gel electrophoresis tank (Marisoru Co.). 20 % polyacrylamide gel was fixed in the tank, and the sample was placed in the sample groove. The voltage was kept at 50 v for 1 hour, and then at 150 v, and the electrophoresis was allowed to proceed until the coloring matter flowed out through the gel; these procedures are defined as SDS-PAGE method throughout the specification and the claims. At the end of the electrophoresis, silver staining was carried out using silver staining kit 161-0443 (Bio-rad Co.) at room temperature to confirm the behavior.

The molecular weight of the LPSs of the present invention was calculated to be 5,000± 1,000 (LPS1 resulting from bacteria 900814-1), and 6,500± 2,500 (LPS2 and LPS3 resulting from bacteria 900814-2 and 900814-3, respectively) in view of the behaviors of the markers for protein m. w. [Pharmacia's LMW kit E: phosphorirase b (94k), albumin (67k), ovalbumin (43k), carbonic anhydrase (30k), trypsin inhibitor (20k), α-lactalbumin (14k)], and those of the markers for peptide m. w. [Pharmacia's 1860-101 m. w. marker: myoglobin (16.9k), myoglobin I & II (14.4k), myoglobin I (8.2k), myoglobin II (6.0k) and myoglobin IV (2.5k). In the same manner as the above, E. coli LPS (0127:B8LPS available from Difco Co.) was found to have dominant m. w. at 40,000 ± 10,000 and 8,000± 4,000.

The stained bands of LPS1, LPS2 and LPS3 in the silver staining are shown in Fig. 1. In Fig. 1, the number 1, 2 and 3 show the stained bands of LPS1, LPS2 and LPS3, respectively. As shown in Fig. 1, LPS1 showed an th r stained band around m. w. 30,000. LPS2 showed another stain d band bridging from 30,000 to 43,000, but it may b said that it contains nly little high mol cular weight substance in vi w of th staining str ngth of the bands at 14,000 or less. Also in view of th sugar cont nt and h x samine content mentioned later, LPS2 the lowest sugar content, and LPS1 has higher sugar c ntent than LPS3. This ord r is believed t be in harmony

with the patterns observed in the electrophoresis. Further, th ratio of LPS cont nt to total dried yield decreases in th order of LPS2, LPS3 and LPS1. Considering the foregoing, it may be estimated that LPS2 comprises relativ ly low molecular weight LPSs, and the content of low molecular w ight LPSs decrease in th rd r of LPS3 and LPS1.

6) Phosphorus content

The captioned content was determined as follows according to the Chen-Toribara method (Chen et al., "Analytical Chemistry", vol. 28, pp. 1756 - 1758, 1956)

LPS1, LPS2 and LPS3 were dissolved in distilled water separately to prepare 20 $\mu\ell$ solutions containing 31.6, 57.6, or 103.6 μg of LPS which were then placed in a small test tube. To the mixture there was added 20 $\mu\ell$ of 50 v/v sulfuric acid followed by heating at 160°C for 2 hours. Then 20 $\mu\ell$ of 50 v/v % perchloric acid was added to the mixture which was then heated on a gas burner for 1 minute to ash. Thereafter, 0.5 mℓ of distilled water and then 0.5 mℓ of a reaction reagent (a portion of the preparation made by mixing 1mℓ of 6N sulfuric acid, 2mℓ of distilled water, 2 ml of 2.5 v/w % ammonium molybdate and 1ml of 10 v/w % of ascorbic acid) were added to the heated mixture which was then allowed to stand for 30 minutes at room temperature. Thereafter the absorption at 820nm (OD_{820nm}) was determined. Here, as the standard sample for the preparation of the calibration curve, potassium didrogen phosphate (manufactured by Wako Jun-yaku Co. in Japan) was diluted with water to prepare 0.5 ml of solutions containing 2.5μg, lug, 0.25μg or 4 μg of phosphorus. In this connection, 1 g of phosphorus corresponds to 4.39 g of potassium didrogen phosphate. The effects observed are shown in Table 4 given below. In the table, the data of absorption are modified by subtracting the values of the control not subjected to the heating from the observed values in order to avoid occurrence of errors due to mixing-in of inorganic phosphorus from, for example, phosphate buffer solution. The P content (μg) is calculated on the basis of the data of absorption. The P content (w/w %) was calculated according to the folloing equation. In the equation, "0.67" is the OD value of 1 μg of the standard phosphorus, and the sample concentration is the proportion of the respective LPSs dissolved in distilled water (mg/ml).

$$\text{P content (w/w \%)} = \frac{\text{Absorption of sample}}{0.67 \times \text{(sample concentration)} \times 0.5}$$

P number is the number of phosphorus per m. w. 5,000 calculated according to the following equation:

$$\text{P number} = \frac{\text{P content (w/w \%)}}{100} \times \frac{5{,}000}{31}$$

Table 4

LPS	Absorption	P content (μg)	P content (w/w %)	P number
1	0.36	0.54	1.7	2± 1
2	0.31	0.46	0.8	1~ 2
3	0.87	1.30	1.3	2± 1

8) Hexosamine content

The captioned content was determined as follows according to the Elson-Morgan method (Library of biochemical experiments. No. 4, pp. 377 - 379, Tokyo Kagaku Dojin Shuppan Co. in Japan).

LPS was dissolved in distilled water to prepare a solution containing 1.58 mg/ml of LPS1, 2.88 mg/ml of LPS2 or 5.18 mg/ml of LPS3, and th respectiv 100 $\mu\ell$ porti ns were placed in a test tub with a screwcap (manufactured by Iwaki Glass Co. in Japan) followed by additi n of 100 $\mu\ell$ f 8N HCℓ th reto, and th mixture was heated at 110°C for 16 hours, and then about 200$\mu\ell$ of 4N NaOH was added to th mixture to bring the pH to 7. A 100$\mu\ell$ portion of the mixture was separated off and placed in another test tube with a screwcap fol-

lowed by addition of 200 μℓ f Reagent A explained below thereto. The mixture was then heated at 105°C for 1.5 h urs, and then cooled with a running water. Next, a 100 μℓ portion of th mixture was separat d off foll wed by additi n of 670 μℓ of a 96 % ethanol and th n 67 μℓ of Reag nt B xplained below, and was then all wed to stand at room temperature for 1 h ur followed by determination of adsorption at 535nm. As th standard sample to prepare th calibrati n curve, 0.20 - 200 μg/mℓ of N-acetyl glucosamin (Wako Jun-yaku Co. in Japan) was used.

Reagent A: prepared by mixing 75μℓ of acetyl acetone and 2.5 mℓ of 1.25 N sodium carbonate

Reagent B: prepared by mixing 1.6g of p-dimethyl benzaldehyde, 30 mℓ of conc. hydrochloric acid and 30 mℓ of 96% ethanol

As a result, the number of hexosaDine in LPS1, LPS2 or LPS3 was 9 ± 1, 7 ± 1 or 5 ± 1 per m. w. 5.000.

9) KDO content

The KDO (2-keto-3-deoxyoctonate) content was determined as follows on the basis of the diphenylamine method (Shaby R. *et al., "lnalytical Biochem."*, 58(1), pp. 123-129, 1974).

A KDO detection reagent was prepared by combining 500 mg of dipenylamine, 5 mℓ of ethanol, 45 mℓ of glacial acetic acid and 50 mℓ of conc. hydrochloric acid (all commercially available from Wako-junyaku Co. in Japan). A 500 μℓ portion of the prepared reagent was combined with 250 μℓ of distilled water containing any of 0.505 mg/mℓ of LPS1, 0.576 mg/mℓ of LPS2 and 0.518 mg/mℓ of LPS3. The resulting mixture was heated in a boiling water bath at 100°C for 33 minutes and then cooled in cooling water at 24.5°C for 30 minutes. The UV absorption of the mixture was determined at 420, 470, 630 and 650 nm to provide data A_{420}, A_{470}, A_{630} and A_{650}, respectively. As the standard sample, there was used 250 μℓ of distilled water containing 0.5 μ mole/mℓ of ammonium salt of KDO (Sigma Co. in U.S.A.). The value S for the test and standard samples was calculated according to the following equation:

$$S = A_{420} - A_{470} + A_{630} - A_{650}$$

The value of the test sample (S_t) was 0.109 for LPS1, 0.078 for LPS2 and 0.099 for LPS 3, whereas that of the standard sample (S_s) was 0.246. The value of distilled water was 0.005. The comparison of these values suggests that LPS1, LPS2 and LPS3 contain 2 ± 1, 1~2 and 2 ± 1 of KOD per m. w. 5,000.

As an example, in the case of LPS1, the KOD content of the solution x (μ mole/ml) may be determined by the equation:

$$\frac{0.5}{0.246} = \frac{x}{0.109}$$

According to the above equation, x is determined to be 0.221. Thus the molar number of KOD contained in 1 mole of LPS1 is determined to be 2.19 according to the following equation on the assumption that 1 mole of LPSi is m. w. 5,000.

$$y = x \times 10^{-6} \times \frac{5,000}{0.505 \times 10^{-3}} = 2.19$$

Illustrative embodiments of preparations containing LPS according to the present invention will be given in the following examples wherein the LPS content is in terms of E. coli LPS calculated according to the limulus test.

Example 2 (tablets)

Wheat LPS	0.04 g
6% HPC lactose	178 g
Talc stearate	8 g
Potato starch	14 g

The above ingredients were mixed and formed into 400 tablets each weighing 0.5 g and containing 0.1 mg of wheat LPS.

Example 3 (solution for internal use)

LPS1	1 mg
Purified water	100 mℓ

Example 4 (ointment)

LPS1	0.1 g
Purified lanolin	80 g
Yellow petrolatum	*ad* 1,000 g

Example 5(injection)

LPS1	0.5 mg
Distilled water for injection	*ad* 1,000 mℓ

Preparation 1 (preparation of B. pertussis LPS)

An experimental B. pertussis solution obtained from Serum Laboratory, a public institute of Chiba prefecture in Japan (2.0×10^{10} cells / ml) was used.

The solution was suspended in sterile water to prepare a suspension containing 25 mg (dry basis) / mℓ of dead cells. To the suspension, there was added an equivalent of a 90% hot phenol solution (68 - 70°C) was added, and the mixture was shaked at 68°C for 1 hr. The mixture was subjected to centrifugation at 8,000 G, 4°C for 20 min. to collect the aqueous layer. Sterile water in the same quantity as of the aqueous layer was added to the remaining phenol, and the mixture was shaked in the same manner as the above. The resulting aqueous layer was combined with the first aqueous layer followed by dialysis in running water overnight, and then the mixture was concentrated to a tenth using a rotary evaporator. The concentrate was subjected to centrifugation at 8,000 G, 4°C for 20 min. The supernatant was separated off, and a small amount of sodium acetate was added thereto. Cold ethanol at 0 - 4°C was added to the mixture in an amount of six times as much as the latter, and the resulting mixture was allowed to stand at -20°C overnight. Then the mixture was subjected to centrifugation at 4,000 G, 4°C for 30 min. to collect the sediment which was subjected to centrifugal washing with ethanol (twice) and acetone (once) followed by drying with an aspirator. The residue was suspended in distilled water to prepare a 20 mg/ml of solution which was then subjected to ultrasonic treatment with a Sonifia 185 (Branson Co. in U.S.A.) (outlet control 5, 15 min., room temperature). The solution was subjected to centrifugation at 2,500 G, 4°C for 10 min. to separate off the supernatant.

The supernatant was treated at 4°C with nucleases, DNase I and Rnase A (both manufactrured by Sigma Co. in U.S.A) for 15 - 16 hrs; totally 10 µg/ml of DNase I and 20 µg/mℓ of Rnase A were used. The same amount of the nucleases as the above were added to the mixture followed by warming at 37°C for 2 hrs and centrifugation at 2,500 G, 4°C for 10 min. to separate off the supernatant.

The supernatant was filtered through a pore size of 0.2 µm using Acrodisc manufactured by Gelman Co. in U.S.A. The filtrate was subjected to molecular sieve (resin: Sepharose 6B manufactured by Pharmacia Co. in U.S.A: column size: 5 cm (i.d.) x 100 cm (length); buffer: 10 mM of Tris-HCℓ / 10 mM of NaCℓ (pH 7.5); flow rate: about 3 mℓ/cm²/hr.). The fractions confirmed to be positive to limulus test with LS-1 kit commercially available from Sei-Kagaku Kogyo Co. in Japan were collected and filtered through a pore size of 0.2 µm using Acrodisc mentioned above. The filtrate was subjected to ion exchange (apparatus: FPLC manufactured by Pharmacia in U.S.A.: resin: mono Q HR 10/10 manufactured by Pharmacia in U.S.A.; buffer: 10 mM of Tris-HCℓ / 10 mM of NaCℓ (pH 7.5); flow rate: 2 mℓ/min.) wherein the filtrate was washed with the buffer for 15 min., then, after the NaCℓ content of th buff r was increas d to 165 mM, for 30 min., th n, for 20 min. while increasing the NaCℓ cont nt to provid a NaCℓ cont nt gradi nt from 165 mM to 1 M, and th n, for 30 min. at th NaCℓ content of 1 M. The fractions confirmed to be positiv to limulus test with LS-1 kit commercially availabl from Sei-Kagaku Kogy Co. in Japan were collected.

The collected fractions wer combined and desalted on a column (resin: Sephad x G-25 fine manufactured by Pharmacia in U.S.A.; column siz : 2 cm (i. d.) x 25 cm (l ngth); eluting agent: distill d water), and then ly philized.

Nucl ic acid is of the greatest possibility of being mixed in th lyophilized sample (4.50 mg). Therefor , th UV absorption curve (200 - 400 nm) was pr pared, and th absorbance at 60 nm was d t rmined.. Th nucleic acid content was calculated to be 1% or less on the basis of the above absorbance in view of the fact that the nucleic acid content was 50 μg/ml in the case where the absorbance was 1. In addition, no apparent evidence showing the presence of a protein was observed in SDS electrophoresis. Thus, considering the detection sensibility, the highest content of proteins which may be mixed in the above lyophilized sample was estimated to be 0 - 3 %. Accordingly, the purity of the above lyophilized sample was estimated to be 96 % or more.

The physical properties of the thus prepared B. pertussis LPS (sometimes referred to only as B. P. LPS) were determined in the same manner as described in Example 1. The results were as follows:

Physical properties of B. pertussis LPS

Molecular weight:	6,000 ± 1,000 (by SDS-PAGE method)
Phosphorus content:	4 per molecular weight of 6,000
Hexosamine content:	12 per molecular weight of 6,000
Fatty acid content:	4 per molecular weight of 6,000
KDO content:	2 ± 1 per molecular weight of 6,000

The physical properties of E. coli LPS (0128: B8 manufactured by Difco Co. in U.S.A.) determined in the same manner as described in Reference Example 1 were as follows:

Physical properties of E. coli LPS

Molecular weight:	40,000 ± 10,000
	8,000 ± 4,000 (by SDS-PAGE method)
Phosphorus content:	12 per molecular weight of 30,000
Hexosamine content:	45 ± 6 per molecular weight of 30,000
Fatty acid content:	18 per molecular weight of 30,000
KDO content:	5 ± 1 per molecular weight of 30,000

Experiment 1 (immunity stimulating effects)

1) Zero point two mℓ of physiological saline containing 1, 10 or 100 μg (in terms of limulus activity) of LPS1, LPS2 or LPS3 was injected into 7 week old C3H/He male mice via caudal vein; each group consisted of two or three mice having an average weight of 25g. One hour later the serun was collected to determine the TNF activity on the basis of the toxicity to L929 cells. The results calculated as an average of two or three per group are shown in Table 5 given below. In the table, parenthesized are the number of the mice used.

Table 5

	TNF activity (units/ml)		
Dose	1 μg	10 μg	100 μg
LPS1	6.15 (3)	25.80 (2)	30.69 (2)
LPS2	1.90 (3)	7.47 (2)	6.57 (2)
LPS3	7.44 (3)	16.19 (2)	34.47 (2)

Experiment 2 (analgesic effects)

To five-membered groups of 7 to 10 week old C3H/He male mice having an average body weight of 28 g, there was orally administrated 200 μl of distilled water containing 0, 1, 5, 25 or 400 μg / mouse of LPS3 or E. coli LPS using a probe. One and a half hours later, 500 μl of 0.7 % acetic acid was given to the mice intraperitoneally over a period of 5 minutes. The frequency of writhing of the respective mice was counted, and the results as shown in Table 6 were recorded (an average of 5 mice in the respective groups). In the table, "-" reflects that the determination was not made at said dose. The writhing inhibition (%) was calculated by the following equation.

{1-[(frequency of writhing at the dose)-(that at 400 μg)]/[(frequency of writhing at 0 μg)-(that at 400 μg)]} x 100

Table 6

LPS dose (µg/ mouse)	LPS3 of the present invention		E. coli LPS	
	Writhing frequency	Writhing inhibition (%)	Writhing frequency	Writhing inhibition (%)
0	18	0	20	0
1	17	10	18	82
5	10	80	-	-
25	7	110	13	64
400	8	100	9	100

Fig. 2 is a graph reflecting the results shown in Table 6. Fig. 2 shows that the writhing inhibition ED_{50} of LPS3 is 2.8 µg / mouse, whereas that of E. coli LPS is 17 µg / mouse. Thus it is supposed that the analgesic effect of LPS3 is about six times as of E. coli LPS.

Experiment 3 (Antiwithdrawal effect - 1)

Molecular sieves were impregnated with morphine HCl available from Takeda Chemical Industries Ltd. in Japan to prepare 12.7 mg of morphine pellet which was then implanted in the back, a little below the neck, of 4 to 5 week old ddY mice (body weight: 20 - 24 g). Two days later, there was given 50 µg / kg of E. coli LPS (6 mice), LPS3 (7 mice) or B.P. LPS prepared in Preparation 1 (6 mice) as a solution in physiological saline. The control group received only physiological saline. One hour later, 10 mg / kg of naloxone was given intraperitoneally, and immediately thereafter the jumping frequency of the mice was counted over a period of 40 minutes to determine the lumping control effects. The results are shown in Table 7. In the table, the figures show the number of the mice concerned. The lumping control effects were evaluated as follows:

The average lumping frequency of the control group (12 mice) per mouse was 62.7 ± 25.5. So, in view of the difference 37 (= 62.5 - 25.5), the case where the jumping frequency was 37 or more was estimated to have "no effect", whereas the case where the frequency was less than 37 was estimated to be "effective".

Table 7

	Jumping inhibition effect	
	Effective	No effect
Physiological saline	1	11
LPS3	7	0
E. coli LPS	3	3
B.P. LPS	4	2

As is apparent in Table 7, the antiwithdrawal Inhibition ratio was only about 8 % in the control grop, whereas the value was 50 %, about 67 % or 100 % in the group to which E. coli LPS, B.P. LPS or LPS3 was given.

Experiment 4 (Antiwithdrawal effect - 2)

In order to determine whether the antiwithdrawal effects of the LPSs of the present invention in intravenous administration are dose-dependent, 12.7 mg of morphine pellet prepared as in Experiment 3 was implanted in the back, a little below the neck, of 4 to 5 week old ddY mice (average body weight: 20 g). Two days later, there was given 0.5 (to 6 mice), 5 (to 6 mice), 15 (to 9 mice), 50 (to 12 mice) or 500 μg / kg (to 6 mice) of LPS3 was given to the mice intravenously as a solution in physiological saline. The control group (10 mice) received only physiological saline. One hour later, 10 mg / kg of naloxone was given intraperitoneally, and immediately thereafter the jumping frequency of the mice was counted over a period of 40 minutes. The results are shown in Table 8 as an average per mouse in the respective groups.

Table 8

Dose of LPS3 (μg/kg)	0	0.5	5	15	50	500
Jumping frequency	69.5	36.8	42.0	16.1	20.5	11.5

Fig. 3 is a graph corresponding to the results given in Table 8.

Experiment 5 (Antiwithdrawal effect - 3)

In ord r to determine whether the antiwithdrawal ff cts of th LPSs of the present inv ntion in intradermal administration are d se-d p nd nt, th procedures of Exp rim nt 4 were followed except that th dose of LPS3 was 50 (to 7 mice) or 500 μg / kg (t 5 mice), and the cotrol group consisted of 8 mice. The results ar shown in Table 9 as an av rag per mous in th respective groups.

Table 9

Dose	Physiological saline	50 µg / kg	500 µg / kg
Frequency	84.7	44	19.8

Fig. 4 is a graph corresponding to the results given in Table 9.

Figs. 3 and 4 clearly show that the anti-withdrawal effects of the LPSs of the present invention are dose-dependent.

Experiment 6 (Antiwithdrawal effect - 4)

In order to determine whether the antiwithdrawal effects of the LPSs of the present invention are dose time-dependent, 12.7 mg of morphine pellet prepared as in experiment 3 was implanted in the back, a little below the neck, of 4 to 5 week old ddY mice (body weight: 20 - 24 g). Two days later, there was given 10 mg / kg of naloxone intraperitoneally. 50 µg /kg of LPS3 was administered to the mice 1 hour (7 mice), 3 hours (8 mice), 8 hours (6 mice) or 18 hours (5 mice) before the administration of naloxone. Immediately after the administration of naloxone, the jumping frequency of the mice was counted over a period of 40 minutes. The control group receiving no LPS3 consisted of 9 mine. The results are shown in Table 10 as an average per mouse in the respective groups.

Table 10

Administration time of LPS3	No dose	Hrs. prior to naloxone administration			
Jumping frequency	65.1	2.7	25.1	33.7	54.6

Fig. 5 is a graph corresponding to the results given in Table 10. Fig. 5 teaches that the LPSs of the present invention have withdrawal-preventive effects, and the maximal preventive effects will be shown when the LPSs are administered immediately before the occurrence of withdrawal symptoms

Dose, interval and toxicity

In view of the nature of immunity stimulators, analgesics and withdrawal agents, and veterinay immunity stimulators, analgesics and withdrawal agents, the dose and the interval of the LPSs of the present invention are of course determined by the doctor or veterinarian in charge individually in view of the age, conditions, etc of the patient and effects of administration. However, it may be said that 1 µg - 100 mg (oral administration), 10 ng - 10 mg (intrav n us administrati n) and 100 ng - 1 mg (percutanous administrati n) are standard singl dose per day to adults (body weight 60 kg). F r veterinary us , about n sixti th of the abov quantities may be giv n per 1 kg f body weight of large-sized animals such as cattl , h rses or th lik . Ab ut twice as much

as the dose to large-sized animals may be given per 1 kg of body weight of medium- or small-siz d animals such as pigs, dogs, cats or the like. Fowls or th lik may receiv twice as much as the dos to medium- r small-sized animals. Th LD_{50} of LPS1, LPS2 and LPS3 in 7 week old C3H/He male mice having an average body weight of 22 g were 150, 180 and 180 μg /mouse according to th Behrens Karber: these valu s are less than 60 % of 300 μg / mouse found for E. coli LPS. Further, E. coli LPS and B. P. LPS had the following LD_{50} (an average of the data on two male BALB/C mice weighing 45 kg on average).

LPS	LD_{50} / kg (mg)	
	i.v.	i.c.
E. coli LPS	3. 4	1 6
B. P. LPS	1 1	3 2

Claims

1. LPS-producing gram-negative small bacilli having the following properties:
   a) Morphological characteristics
      1) Small rod
      2) No Motility
      3) Gram stain: -
   b) Growth
      1) Standard agar medium: a yellow to creamy round opaque colony is formed.
      2) SS agar medium: A white translucent colony is formed.
      3) TSI agar medium: No change is found on the slant, but the higher layer changes to yellow. Gas if produced.
   c) Physiological characteristics
      1) Voges-Proskauer reaction: +
      2) Indole production: -
      3) Hydrogen sulfide production: -
      4) Utilization of citrate: +
      5) Urease: -
      6) Oxidase: -
      7) O-F test: +
   d) Utilization of carbon sources
      1) Lactose: +
      2) Adonitol: -
      3) Rhamnose: +
      4) Mannitol: +
      5) Esculin: +
      6) Inositol: -
      7) Sorbitol: +
      8) Arabinose: +
      9) Raffinose: +
      10) Sucrose: +
   e) Others
      1) Lysin decarboxylase: -
      2) Utilization of malonate: -
      3) Arginine dihydroxylase: -
      4) Phenylalanin deaminase: -
      5) Ornithin decarboxylas : -

2. LPS-producing gram-negative small bacilli having th f llowing properties:

a) Morphological characteristics
1) Small rod
2) No M tility
3) Gram stain: -
b) Growth
1) Standard agar medium: a creamy opaque colony is formed.
2) SS agar medium: A red opaque colony is formed.
3) TSI agar medium: No change is found on the slant, but
c) Physiological characteristics
1) Voges-Proskauer reaction: +
2) Indole production: -
3) Hydrogen sulfide production: -
4) Utilization of citrate: +
5) Urease: -
6) Oxidase: -
7) O-F test: +
d) Utilization of carbon sources
1) Lactose: +
2) Adonitol: -
3) Rhamnose: +
4) Mannitol: +
5) Esculin: +
6) Inositol: -
7) Sorbitol: +
8) Arabinose: +
9) Raffinose: +
10) Sucrose: +
e) Others
1) Lysin decarboxylase: -
2) Utilization of malonate: +
3) Arginine dihydroxylase: +
4) Phenylalanine deaminase: -
5) Ornithine decarboxylase: +

3. LPS-producing gram-negative small bacilli having the following properties:
a) Morphological characteristics
1) Small rod
2) No Motility
3) Gram stain: -
b) Growth
1) Standard agar medium: A yellow round translucent colony is formed.
2) SS agar medium: No colony is formed.
3) TSI agar medium: No change is found on the slant, but the higher layer changes to yellow. Gas in not produced.
c) Physiological characteristics
1) Voges-Proskauer reaction: +
2) Indole production: -
3) Hydrogen sulfide production: -
4) Utilization of citrate: +
5) Urease: -
6) Oxidase: -
7) O-F test: +
d) Utilization of carbon sources
1) Lactose: +
2) Ad nitol: -
3) Rhamn se: +
4) Mannitol: +
5) Esculin: +

6) Inositol: -
7) Sorbitol: +
8) Arabinose: +
9) Raffinose: -
10) Sucrose: +

e) Others

1) Lysin decarboxylase: -
2) Utillzation of malonate: +
3) Arginine dihydroxylase: -
4) PhenyLalanine deaminase: -
5) Ornithine decarboxylase: -

4. LPS provided by the bacilli of Claim 1 and exhibiting the dominant molecular weight of 5,000 ± 1,000 as determined by SDS-PAGE method, having 2 ± 1 phophorus, 9 ± 1 hexosamines and 2 ± 1 KDO per molecular weight of 5,000.

5. LPS provided by the bacilli of Claim 2 and exhibiting the dominant molecular weight of 6,500 ± 2,500 as determined by SDS-PAGE method, having 1 - 2 phophorus, 7 ± 1 hexosamines and 1 - 2 KDO per molecular weight of 5,000.

6. LPS provided by the bacilli of Claim 3 and exhibiting the dominant molecular weight of 6,500 ± 2,500 as determined by SDS-PAGE method, having 2 ± 1 phophorus, 5 ± 1 hexosamines and 2 ± 1 KDO per molecular weight of 5,000.

7. An immunity stimulator containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

8. An analgesic containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

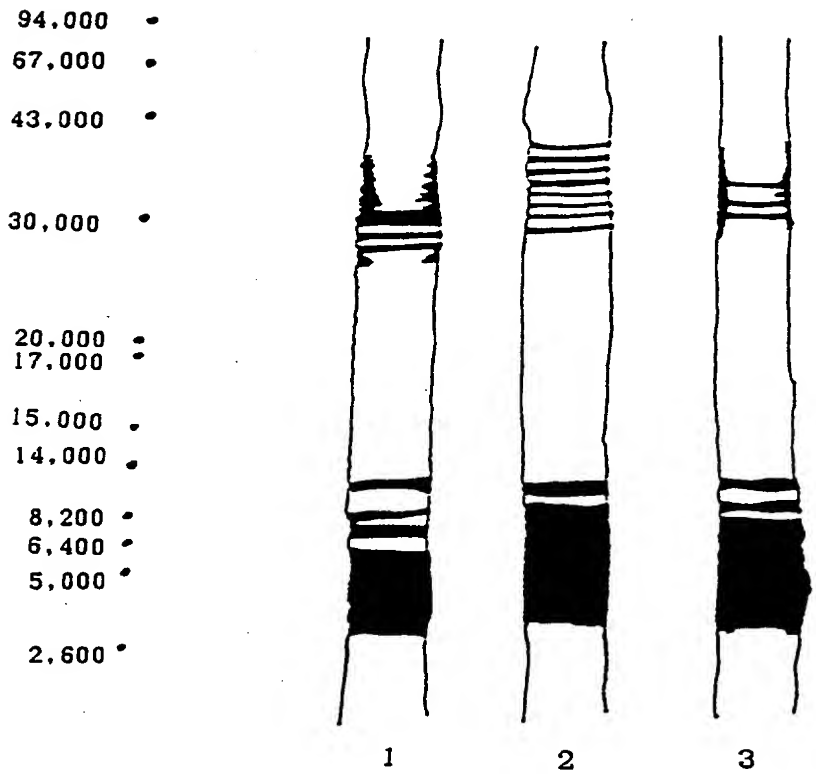
9. An antiwithdrawal agent containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

10. A veterinay immunity stimulator containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

11. A veterinay analgesic containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

12. A veterinary antiwithdrawal agent containing LPS selected from the group consisting of the LPSs of Claims 4 - 6 and mixtures thereof.

FIG. 1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67,000
43,000
30,000
20,000
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8,200
6,400
5,000
2,600
1
2
3

F I G. 2

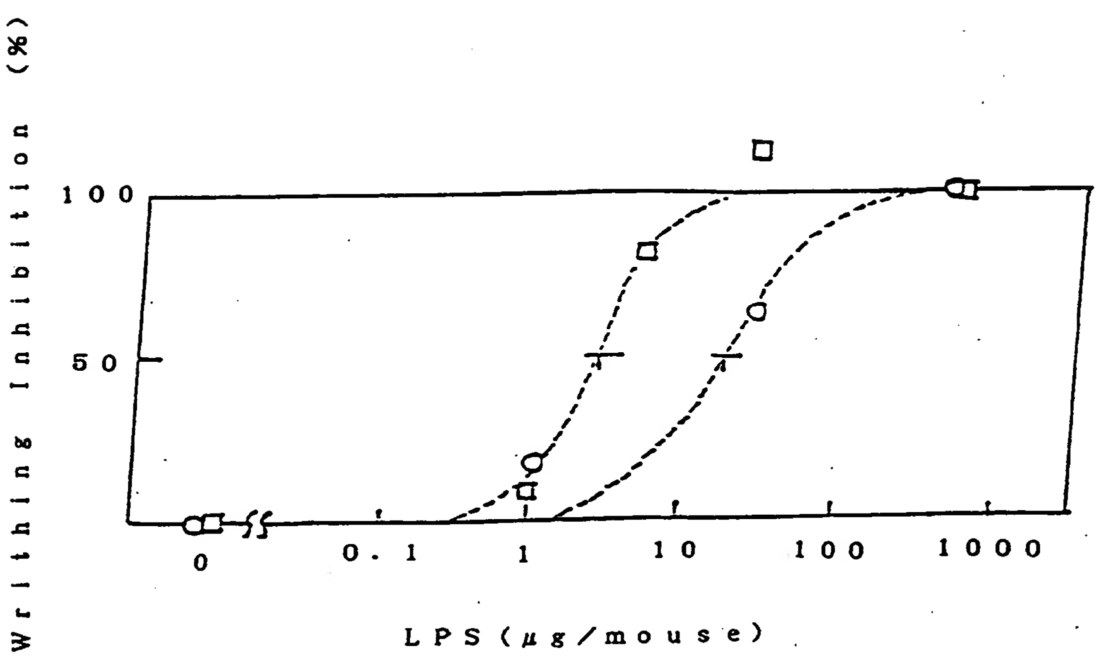
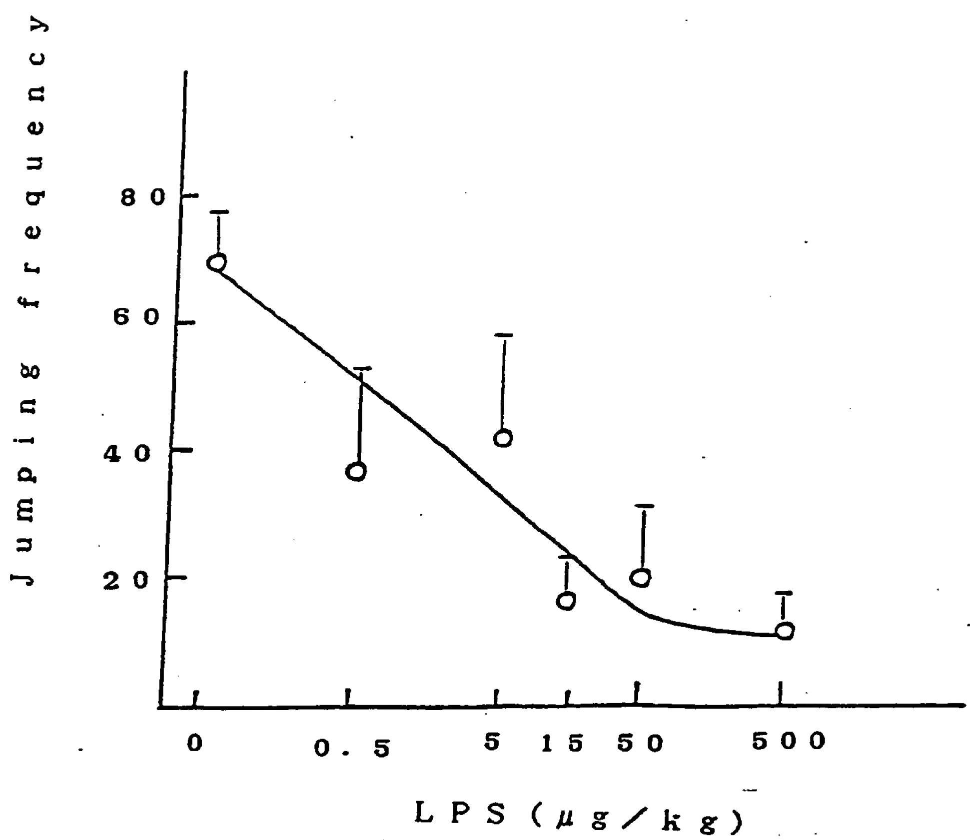


FIG. 3



F I G. 4

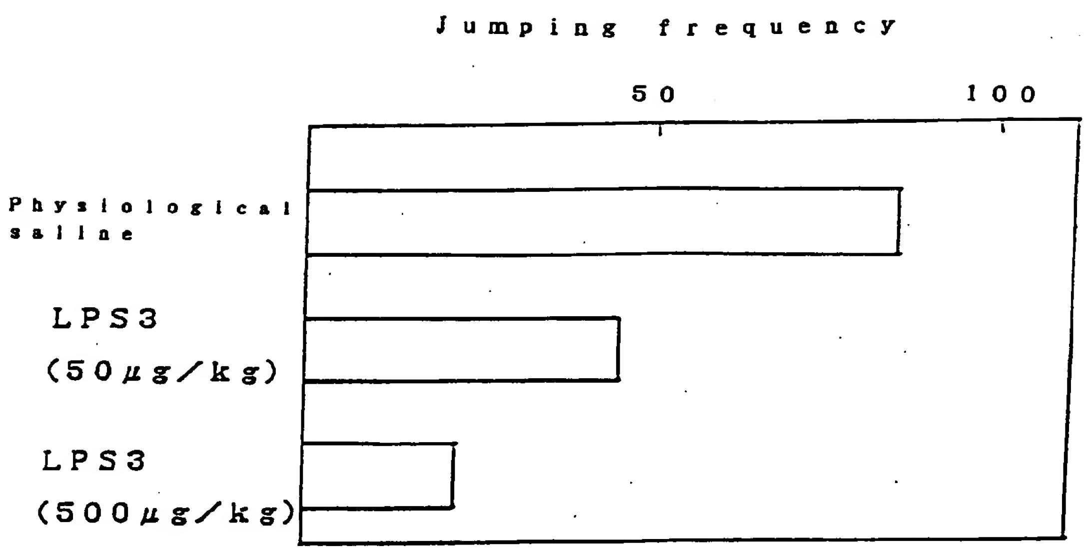
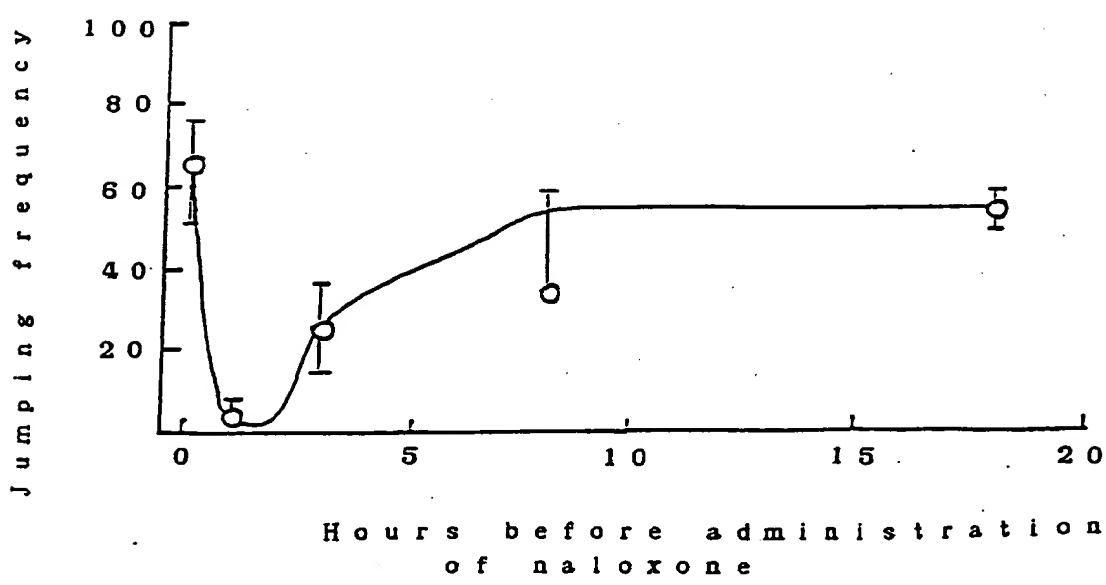

Jumping frequency
50
100
Physiological saline
LPS3 (50μg/kg)
LPS3 (500μg/kg)

FIG. 5



Jumping frequency
100
80
60
40
20
0
5
10
15
20
Hours before administration of naloxone

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(54) **LPS-Containing analgesics and veterinary analgesics.**

(57) An analgesic composition comprising an effective amount of at least one member of LPS whose macrophage activation ED_{50} is 0.4 - 100 ng/ml of culture solution in terms of its limulus test-positive LPS content observed on a sigmoid curve prepared by determining the ability of the LPS to activate the TNF productivity of macrophage cultured *in vitro*, and plotting the macrophage activation ability (%) along the axis of ordinate wherein the ability is estimated to be 0 % in the case where it corresponds to the quantity of TNF produced by macrophage with no LPS added thereto, and 100 % is assigned to the macrophage activation ability which provides the maximal and constant quantity of TNF produced by the macrophage and plotting the limulus test-positive LPS content of the LPS along the axis of abscissa on a logarithmic scale, in admixture with a pharmaceutically or veterinarily acceptable carrier, such that when administered to an animal, high cholesterol level of said animal is prevented or cured ; and

a method of treating pain of an animal comprising administration to said animal an amount of the above composition effective to prevent or cure the pain of said animal.

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 40 2276
Page 1

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
E	EP-A-0 462 022 (CHIBA FLOUR MILLING CO.LTD) * the whole document * ---	1-2	A61K37/20
E	EP-A-0 462 021 (CHIBA FLOUR MILLING LTD CO.) * the whole document * ---	1-2	
E	EP-A-0 462 020 (CHIBA FLOUR MILLING LTD CO.) * the whole document * ---	1-2	
X	CHEMICAL ABSTRACTS, vol. 108, no. 25, 20 June 1988, Columbus, Ohio, US; abstract no. 220113, NAKAMURA, H. ET AL 'Interleukin-1 induces analgesia in mice by a central action' page 430 ;column 1 ; & Eur. J. Pharmacol. 1988 149(1-2) pages 49-54 * abstract * ---	1-2	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
X	CHEMICAL ABSTRACTS, vol. 109, no. 11, 12 September 1988, Columbus, Ohio, US; abstract no. 85526, KOTANI, S. ET AL 'Synthetic lipid A analogs as a candidate for useful immunomodulators' page 2 ;column 2 ; & Prog. Leukocyte Biol. 1987 6 (immunopharmacol. infect. dis.) pages 223-234 * abstract * --- -/--	1-2	A61K C12P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	04 JANUARY 1993	FERNANDEZ Y BRA F.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 91 40 2276
Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
T	CHEMICAL ABSTRACTS, vol. 117, no. 3, 20 July 1992, Columbus, Ohio, US; abstract no. 20426, OKUTOMI, T. ET AL 'Homeostasis as regulated by activated macrophage. IV. Analgesic effect of LPSw, a lipopolysaccharide of wheat flour.' page 84 ;column 2 ; & Chem. Pharm. Bull. 1992 40(4) pages 1001-1003 * abstract * -----	1-2	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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(54) **ADDITIVES FOR CRUSTACEAN OR FISH FEEDS AND FEEDS**

(57) A feedstuff additive for crustaceans and fishes, characterized in that it is prepared from gram-negative bacteria, that it has a molecular weight of 5000 ± 2000 as measured by SDS-PAGE method using a protein marker, that it is substantially free of high molecular weight lipopolysaccharide, that it contains a low molecular weight lipopolysaccharide as an effective component and that it is capable of activating immunity and preventing infection in crustaceans or fishes ; and a feed for crustaceans and fishes comprising the above feedstuff additive.

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TECHNICAL FIELD

[0001] The present invention relates to a feedstuff additive for crustaceans or fishes, and a feed containing the feedstuff additive, and more particularly to a feedstuff additive which shows significant effects of activating immunity and preventing infection and to a feed containing the same in a suitable proportion.

BACKGROUND ART

[0002] Recent years have seen development of aquiculture of crustaceans and fishes. Attendant on the development is a great economical damage in the culture industry due to outbreaks of bacterial or viral diseases of crustaceans and fishes. Diseases of crustaceans and fishes often occurring include acute viremia of kuruma prawns (Penaeus japonicus), vibriosis thereof, pseudotuberculosis of yellowtails, enterococcus diseases thereof, cold-water disease of sweet fishes (ayu), Pseudomonas diseases thereof, iridovirus diseases of red sea breams, Seriola dumerili, yellowtails or the like which have economically damaged the culture industry. Of these diseases, bacterial diseases have been treated with antibiotics or synthetic antibacterial agents as a curative agent. However, with the advent of antibiotic-resistant bacteria, satisfactory curative effects have not been achieved. Further, a problem of public health hazards has been raised because of the medicinal agent remaining in crustaceans and fishes. Consequently, there is a strong demand for preventive measures not depending on chemotherapy. On the other hand, vaccines and curative agents have not been developed against viral diseases of crustaceans and fishes and viral diseases still often occur.

[0003] The use of polysaccharides is already known to immunopotentiate crustaceans and fishes and to prevent infectious diseases thereof. These polysaccharides include, for example, peptidoglycan derived from Bifidobacterium thermophilum (Patent No.2547371), cell wall-forming component of gram-positive bacteria like bacteria of genus Bacillus (JP-B-3-173826) and β-1,3-glucan derived from Schizophyllum commune (JP-B-6-65649). It was already reported that high molecular weight lipopolysaccharides activate the immune function of fishes and animals (Salati, F. and R. Kusuda, Society Journal, Japanese Society of Science of Fisheries, vol.53, pp.201 to 204, 1987 and Odean, M.J. et al., Infection and Immunity, vol.58, pp.427 to 432, 1990).

[0004] On the other hand, the low molecular weight lipopolysaccharide of the present invention (hereinafter referred to as "low molecular weight LPS") is different in basic structure and components from the peptidoglycan derived from gram-positive bacteria, cell wall-forming component and β -1,3-glucan derived from a mushroom. The low molecular weight LPS of the invention comprises three components, i.e. a specific lipid A, an oligosaccharide with covalent bond therewith called R core and O specific polysaccharide. The low molecular weight LPS of the invention is known as an immunopotentiator for animals because of its ability to increase the tumor necrosis factor (TNF)-producing effect, but is not known at all to have an activity of preventing infectious diseases of crustaceans and fishes. The high molecular weight lipopolysaccharides (LPSs) used in the researches heretofore reported are those with a markedly high molecular weight as high as 1 million to 10 millions and are of high toxicity. Consequently, when applied to crustaceans and fishes for a long period, such high molecular weight LPS is unable to activate the immune function all the time. The above-mentioned known substances have a high molecular weight and need to be orally administered in a large quantity because of their poor absorption through the intestinal tract. Consequently, a long-period intake of them frequently results in impairment of immune function.

[0005] As described above, a variety of infectious diseases often occur in crustaceans and fishes. Some of these diseases are lethal and may result in great economic damage. The background to be noted is that the immune function of crustaceans and fishes is deteriorated because they are bred in an overcrowded area under a limited environment. Various substances were used to reactivate their impaired immune system. On the other hand, crustaceans have no ability to produce an antibody nor lymphocyte, neutrophile or basophile as found in a vertebrate. Fishes have a limited ability to produce an antibody and its production of antibody is greatly affected by the temperature of water because they are cold-blooded animals so that such immune system is not sufficiently functioned. In other words, substantial difference exists in defensive mechanism between these oceanic organisms and mammals (Fish Pathology, 30(2), 141-150, June in 1995). Consequently some of the substances are not usable in-situ in breeding oceanic organisms because of high toxicity like conventional LPSs, and most of them are impaired in the immune system by intake of the LPSs for a prolonged period.

[0006] An object of the present invention is to provide a safe feedstuff additive for culture or breeding of crustaceans and fishes, the feedstuff additive being capable of preventing infectious diseases even in a small amount by properly activating their intrinsic immune function, and being free from problems of public health hazards such as the feedstuff additive remaining in crustaceans and fishes.

DISCLOSURE OF THE INVENTION

[0007] The present invention provides a feedstuff additive for crustaceans and fishes, characterized in that it is prepared from gram-negative bacteria, that it has a molecular weight of 5000 ± 2000 as measured by SDS-PAGE method using a protein marker, that it is substantially free of high molecular weight lipopolysaccharide, and that it contains a low molecular weight lipopolysaccharide as an ffective component and that it is capabl of activating immunity or preventing infection in crustaceans or fishes; and a feed for crustaceans or fishes which feed is characterized in that it contains the feedstuff additive.

[0008] The present invention also provides a feedstuff additive for crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

[0009] The present invention also provides use of the low molecular weight lipopolysaccharide for the preparation of a feedstuff additive for crustaceans or fishes.

[0010] The present invention also provides a method of activating immunity or preventing infection in crustaceans and fishes comprising administering an effective amount of the low molecular weight lipopolysaccharide to crustaceans or fishes.

[0011] The present invention also provides an agent for preventing the perish of crustaceans or fishes comprising the low molecular weight lipopolysaccharide as an effective component.

[0012] The present invention also provides an agent for preventing the perish of crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

[0013] The present invention also provides use of the low molecular weight lipopolysaccharide of for the preparation of an agent for preventing the perish of crustaceans or fishes.

[0014] The present invention also provides a method of preventing the perish of crustaceans or fishes comprising administering an effective amount of the low molecular weight lipopolysacchride to crustaceans or fishes.

[0015] The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are those pertaining to genus Pantoea.

[0016] The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are Pantoea agglomerans.

[0017] The present invention also provides a feed for crustaceans or fishes comprising the feedstuff additive.

[0018] The present invention also provides a feed for crustaceans or fishes comprising the agent for preventing the perish.

[0019] The present invention also provides a method of breeding crustaceans or fishes comprising administering the feed to crustaceans or fishes.

[0020] The feedstuff additive of the invention is prepared from gram-negative bacteria by purification, e.g. according to the method disclosed in JP-A-8-198902. The present inventors prepared a feed containing a low molecular weight LPS having a molecular weight of 5000 ± 2000. When the feed was supplied to crustaceans and fishes, it was found that the feed prevented viral or bacterial infectious diseases and protected them against decease by activation of the intrinsic immune function. The present invention was accomplished based on this finding.

[0021] The low molecular weight LPS of the present invention is, as described above, a lipopolysaccharide having a molecular weight of 5000 ± 2000 which is prepared from gram-negative bacteria, e.g. according to the method disclosed in JP-A-8-198902. The LPS of this invention is characterized in that the LPS is pronouncedly safer for crustaceans or fishes and can produce a significantly higher effect of activating immunity and a higher effect of preventing infection and decease than conventional LPSs (with a molecular weight of 1 million to 10 millions).

[0022] In the present invention, the term "substantially free of high molecular weight lipopolysaccharide" means "not containing lipopolysaccharide having a molecular weight of at least 8,000".

[0023] The gram-negative bacteria for use in the invention include, for example, those pertaining to genera Pantoea, Salmonella, Aeromonas, Serratia and Enterobacter, and further include those described in JP-A-4-99481. Among useful gram-negative bacteria, those of Pantoea are preferred and those of Pantoea agglomerans are more preferred.

[0024] The low molecular weight LPS of the present invention can be prepared by a method comprising incubating gram-negative bacteria or the like in the conventional manner, collecting the cultured bacteria from the culture medium, extracting the collected bacteria by conventional methods, such as hot phenol method (edited by O. Westphal, Methods in Carbohydrate Chemistry, vol. 5, p.83, Academic Press, 1965) and purifying the extract with an anion exchange resin. More specifically, the method comprises suspending bacteria in distilled water, adding the suspension to a mixture of distilled water and an equal volume of hot phenol, stirring the mixture, centrifuging the mixture to recover the aqueous layer, dialyzing the aqueous layer to remove the phenol, concentrating the aqueous layer by ultrafiltration to obtain crude LPS fractions, purifying the fractions by conventional anion exchange chromatography (e.g. using mono Q-Sepharose or Q-Sepharose) and desalting the same in the conventional manner.

[0025] The purified LPS thus obtained is substantially identical with the LPSs having a molecular weight of about 5,000 to about 6,000 as disclosed in JP-A-4-187640, JP-A-4-49240, JP-A-4-99481 and JP-A-5-155778. The purified

LPS is subjected to gel filtration in the presence of a surface-active agent such as sodium deoxycholate to recover only low molecular weight LPS-containing fractions, whereby only a highly purified low molecular weight LPS is obtained by removal of the high molecular weight LPS from the fractions. The procedure of gel filtration in the presence of a surface-active agent is carried out to more highly purify the LPSs having a molecular w ight of about 5,000 to about 6,000 which are disclosed in JP-A-4-187640, JP-A-4-49240 and JP-A-5-155778, whereby th high molecular weight LPS is completely removed from the fractions.

[0026] The term "crustaceans" used herein refers to all of lobsters, shrimps or prawns such as kuruma prawn (Penaeus japonicus), ushi prawn (Penaeus monodon), Yellow Sea prawn (Penaeus chinensis) and banana prawn (Penaeus morguiensis), and all of crabs such as Portunus trituberculatus and Chinese mitten crab, preferably lobsters, shrimps or prawns, more preferably prawns. The term "fishes" used herein include all of fishes such as yellowtail, globefish, real sea bream, flatfish, eel and rainbow trout. The infectious diseases referred to herein include acute viremia of crustaceans, their vivrio diseases, parasitosis such as Bpistylis sp., Zoothamnium sp. or mycosis such as Lagenidium sp., Siropidium sp.; iridovirus infectious diseases of fishes, their rhabdovirus diseases, neuronecrosis, infectious hemopoietic organ necrosis, pseudotuberculosis, streptococcic diseases, enterococcus diseases, vivrio diseases, cold-water disease, Pseudomonas diseases, gliding-bacteria diseases and Saprolegnia diseases, and all of infectious diseases caused by viruses, mycoplasmas, bacteria, fungi and parasites among which the feedstuff additive and feed of the invention can be more effectively used for viremia of crustaceans, and fishes' diseases such as streptococcic diseases, enterococcus diseases and vivrio diseases.

[0027] The low molecular weight LPS of the present invention can be used as a feed additive for crustaceans and fishes, and for this purpose, may be used as it is or as mixed with conventional carriers, stabilizers and the like and optionally with vitamins, amino acids, minerals and like nutrients, antioxidants, antibiotics, antibacterial agents and other additives. The feed additive is prepared in a suitable form such as powders, granules, pellets or suspensions. The feed additive may be supplied to crustaceans or fishes, alone or in mixture with a feed. For prevention of diseases, the feed additive may be supplied together with the feed at all times or at a latter half of feeding time.

[0028] The feeds of the present invention are not specifically limited but can be any of powdery feeds, solid feeds, moist pellet feeds, dry pellet feeds, extruder pellet feeds and live baits.

[0029] The proportion of the low molecular weight LPS in the feed of the invention can be selected from a wide range and is preferably 0.000001 to 0.001% by weight, more preferably 0.00002 to 0.00005% by weight to which its proportion is not limited. The amount of the low molecular weight LPS to be used can be suitably determined. For example, the LPS is applied at a daily dose of 1 to 100 µm, preferably 10 to 20 µg, per kilogram of the body weight of crustaceans or fishes to which, however, the dose is not limited.

BEST MODE OF CARRYING OUT THE INVENTION

[0030] The present invention will be described in detail with reference to the following Examples to which, however, the invention is not limited. Low molecular weight LPS used in Examples is LPS having a molecular weight of about 5,000, and high molecular weight LPS is LPS having a molecular weight of about 8,000 to 50,000.

<u>Reference Example 1</u> (Preparation of low molecular weight LPS)

[0031] A 10 g quantity of tryptone (product of DIFCO CO.), 5 g of yeast extract (product of DIFCO CO.) and 10 g of NaCl (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) were added to 1 liter of distilled water. The suspension was adjusted to a pH of 7.5 with NaOH and was sterilized in an autoclave. A single colony was separated from Pantoea agglomerance-carrying bacteria maintained at -80°C and was inoculated in a 500 ml-vol. Sakaguchi flask holding 100 ml of a culture medium containing sterile glucose (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) at a proportion of 0.1% (hereinafter referred to as L-broth medium). Then the cells were subjected to shake culture at 35°C overnight. The cultured cells were inoculated in its entirety in a 3 liter-vol. Sakaguchi flask holding 1,000 ml of L-broth medium and were further cultivated in the same manner as above.

[0032] The cultured cells were inoculated in a 10-liter vol. desk fermenter (product of MARUBISHI BIOENGI CO.) holding 7 liters of L-broth medium and were subjected to aeration culture under the same conditions. The cells were collected to recover about 70 g of wet bacteria and were freeze-stored. About 70 g of freeze-stored cells were suspended in 500 ml of distilled water. A 500-ml quantity of 90% hot phenol was added to the suspension. The mixture was stirred at 65 to 70°C for 20 minutes and was cooled. The mixture was centrifuged at 10,000 G and 4°C for 20 minutes to recover the aqueous layer. The phenol layer was treated in the same manner as above. Then the two aqueous layers thus obtained were combined and dialyzed overnight to remove the phenol. The inner solution was concentrated by ultrafiltration in a 2 atom. nitrogen gas using an ultrafiltration device (product of ADVANTEC TOYO CO., K-200) with a membrane filter by cutting off molecular w ight 200,000.

[0033] The lyophilized product of crude LPS thus obtained was dissolved in distilled water, the filter was sterilized,

a buffer was added, and th solution was subjected to anion exchange chromatography (product of PHARMACIA Co., Q-Sepharos first flow). Th specim n solution was pass d through the column using a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl to elute a limulus active fraction with 200 to 400 mM NaCl/10 mM Tris-HCL (pH 7.5). The eluate was subjected to ultrafiltration under th same conditions as above for desalting and concentration and was lyophilized to obtain about 300 mg of purified LPS from about 70 g of wet bacteria.

[0034] The obtained purified LPS (100 mg) was dissolved in a solubilizing buffer [comprising 3% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA-2Na and 20 mM Tris-hydrochloric acid, pH 8.3]. The purified LPS solution (20 ml) was gently placed over Sephacryl S-200 HR column (product of PHARMACIA CO.). Then, 800 ml (50 hours) of the solution was eluted with an eluting buffer [comprising 0.25% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA and 10 mM Tris hydrochoric acid, pH 8.3] at a flow velocity of 16 ml/hr.

[0035] The obtained eluate was fractionated by a fraction collector (product of ADVANTEC CO., trade name SF 2120) under control of flow velocity using a perista-pump PI (product of PHARMACIA CO.). A first 240-ml portion (24-fraction portion) was cast away. Thereafter the residue was fractionated into 80 fractions at 10 ml/fraction. The saccharide in the eluted fractions was quantitatively determined using the base solution or diluted solution by phenol/sulfuric acid method (Sakuzo FUKUI, "Method of Quantitative Determination of Reducing Sugar", 2nd ed., pp. 50 to 52, Gakkai Shuppan Center, 1990) to check the elution state. The fraction pattern of LPS was investigated by SDS-PAGE method using 0.5 ml of each of fractions 37 to 55 among the fractions presumably having LPS (fractions 30 to 60).

[0036] The result of investigation demonstrates that the fractions 45 to 55 contained only low molecular weight LPS (m.w. about 5000) and that fractions 37 to 44 contained both low molecular weight LPS and high molecular weight LPS. The low molecular weight LPS fractions of fractions 45 to 55 were further purified is follows.

[0037] The fractions was mixed, lyophilized and suspended in ethanol. The suspension was centrifuged to remove the deoxycholic acid soluble in ethanol and to recover a low molecular weight LPS in insoluble fractions. The ethanol treatment of the low molecular weight LPS fractions was further repeated twice, followed by removal of deoxycholic acid. The obtained LPS was suspended in 70% ethanol again, and the buffer component was removed by centrifugation. The same procedure was repeated three times for recovery of low molecular weight LPS in the insoluble fractions, followed by lyophilization, whereby about 20 mg of purified low molecular weight LPS was produced.

Example 1 (Safety of low molecular weight LPS in crustaceans)

[0038] Kuruma prawns having an average weight of 20 g were divided into 5 groups of each 20 prawns. The low molecular weight LPS of the present invention was intramascularly administered to the third abdominal segment of prawns in Groups 1 and 2 at a dose of 50 mg and 100 mg, respectively per kilogram of the prawn's weight. On the other hand, a conventional high molecular weight LPS (derived from E. coli, E. coli 0111 manufactured by DIFCO CO.) was intramascularly administered to the third abdominal segment of prawns in Groups 3 and 4 at a dose of 10 mg and 20 mg, respectively per kilogram of the prawn's weight. Group 5 received a physiological saline free of LPS. The life or death of prawns up to 120 hours after administration was checked to determine a mortality. The results are shown in Table 1.

Table 1

Group	number of perish / number tested	mortality (%)
Group 1 low MW LPS 50 mg/kg	0/20	0
Group 2 low MW LPS 100 mg/kg	0/20	0
Group 3 high MW LPS 10 mg/kg	13/20	65
Group 4 high MW LPS 20 mg/kg	20/20	100
Group 5 physiological saline	0/20	0

[0039] As apparent from Table 1, a mortality of prawns in the groups receiving 10 mg or 20 mg of high molecular weight LPS was 65 or 100%, respectively, whereas no prawn died in the groups receiving 50 mg and 100 mg of low molecular weight LPS. It is clear from the above data that low molecular weight LPSs are significantly safe for prawns as compared with conventional high molecular weight LPSs.

Example 2 (Safety of low molecular weight LPS in fishes)

[0040] Black carps having an average weight of 85 g were divided into 3 groups of each 40 carps. Th low molecular weight LPS of the present inv ntion was intramascularly administered to the dorsal region of black carps in Group 1 at a dose of 100 mg per kilogram of the carp's weight. On the other hand, a conventional high molecular weight LPS (trade name E. coli 0111 manufactured by DIFCO CO.) was intramascularly administ red to the dorsal region of black carps in Group 2 at a dose of 20 mg per kilogram of the carp's weight. Group 3 received a physiological saline free of LPS. The life or death of black carps up to 120 hours after administration was checked to determine a mortality. The results are shown in Table 2.

Table 2

Group	number of perish / number tested	mortality (%)
Group 1 low MW LPS 100 mg/kg	0/40	0
Group 2 high MW LPS 20 mg/kg	34/40	85
Group 3 physiological saline	0/40	0

[0041] As apparent from Table 2, a mortality of black carps was 85% in the group receiving 20 mg of high molecular weight LPS, whereas no black carp died in the group receiving 100 mg of low molecular weight LPS. It is clear from the above data that the low molecular weight LPS of the present invention is significantly safe for fishes as compared with conventional high molecular weight LPS.

Example 3 (Activity of activating phagocytosis in hemocyte of crustaceans)

[0042] Kuruma prawns having an average weight of 20 g were divided into 6 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 µg, respectively per kilogram of prawn's weight. On the other hand, Group 4 received a high molecular weight LPS as admixed with a feed at a daily dose of 100 µg, and Group 5 received the same at a daily dose of 1000 µg, per kilogram of prawn's weight. The feeds were given for 7 days. Group 6 was given a feed free of LPS. On day 0, day 1, day 5 and day 7 after supply of the feeds, the blood was collected from the thorax recess of prawns using a syringe holding a K-199 culture medium containing L-cystein as an anticoagulant. Hemocyte cells were obtained by centrifugation. The obtained cells (1×10^5 cells per microliter of the suspension) were mixed with 1×10^8 latex beads (1.986 µm in diameter) and were reacted at 25°C for 30 minutes. After fixing the reaction mixture with glutaraldehyde, it was air-dried. Then the mixture was subjected to giemsa staining and was fixed to a slide glass with Eukitt. The same procedure was repeated to obtain five samples per prawn. The hemocyte cells (200 cells per sample) were observed at random under an epi-fluorescent microscope to determine the phagocytosis ratio of latex beads in hemocyte and the number of latex beads phagocytized into one cell of hemocyte. Then the phagocytosis index was calculated by the following equation.

Phagocytosis ratio= [number of hemocyte cells taking beads/total number of hemocyte cells observed] × 100.

Average number of beads taken by hemocyte cells =
number of beads taken by hemocyte cells/number of hemocyte cells taking beads.

Phagocytosis index= [number of hemocyte cells taking beads/total number of hemocyte cells observed]
× [number of beads taken by hemocyte cells/total number of hemocyte cells observed] × 100.

Test results: The biophylaxis of crustaceans involves a cell factor and a liquid factor. The phagocytosis of foreign particles in hemocyte is deeply concerned with the former. When the phagocytosis of foreign particles in prawn's hemocyte is assessed, it is clarified whether the defensive mechanism of prawns is activated. [Yukinori TAKAHASHI et al, Research of Fish Diseases, 30 (2), pp.141 to 150, (1995)]. In view of said theory, the phagocytosis index was determined on day 0, day 1, day 5 and day 7 after supply of feeds for the groups receiving high molecular weight LPSs and the groups receiving the low molecular weight LPSs. The results were tabulated in Table 3.

Table 3

Group	Phagocytosis index of hemocyt	
	0	1 day
Group 1 low MW LPS 20 µg/kg	0.9±0.18	2.1±0.61 *2
Group 2 low MW LPS 40 µg/kg	0.9±0.18	3.3±1.16 *2
Group 3 low MW LPS 100 µg/kg	0.9±0.18	3.8±1.00 *2
Group 4 high MW LPS 100 µg/kg	0.9±0.18	0.7±0.31
Group 5 high MW LPS 1000 µg/kg	0.9±0.18	1.1±0.63
Group 6 feed free of LPS	0.9±0.18	0.5±0.24
	5 days	7 days
Group 1 low MW LPS 20 µg/kg	3.2±0.71 *2	8.4±1.37 *2
Group 2 low MW LPS 40 µg/kg	4.5±0.75 *2	3.7±1.02 *2
Group 3 low MW LPS 100 µg/kg	3.1±0.94 *2	2.8±0.70 *1
Group 4 high MW LPS 100 µg/kg	0.7±0.82	1.2±0.44
Group 5 high MW LPS 1000 µg/kg	2.1±0.58 *1	2.9±0.68 *1
Group 6 feed free of LPS	0.7±0.5	1.1±0.56

*1: significant difference between this group and Group 6 ($P<0.05$)
*2: significant difference between this group and Group 6 ($P<0.01$)

[0043] As apparent from Table 3, the groups receiving the low molecular weight LPSs (present invention) showed a higher phagocytosis index in hemocyte of prawns than Group 6 and a significant difference in this index from Group 6 ($P<0.01$, $P<0.05$). The group receiving 100 µg of conventional high molecular weight LPS was unable to increase the phagocytosis index in hemocyte of prawns after 1, 5 and 7 days. However, the group receiving 1000 µg of conventional high molecular weight LPS showed a significantly higher phagocytosis index in hemocyte of prawns ($P<0.05$) than Group 6 after 5 and 7 days. The above data show that the low molecular weight LPSs of the present invention can activate the defensive mechanism such as phagocytosis in hemocyte of prawns even when used in an extremely smaller amount than the high molecular weight LPSs.

Example 4 (Activity of activating phenol oxidase in hemocyte of crustaceans)

[0044] Kuruma prawns having an average weight of 20 g were divided into 6 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 µg, respectively per kilogram of prawn's weight. Group 4 received a high molecular weight LPS as admixed with a feed at a daily dose of 100 µg, and Group 5 received the same as admixed with a feed at a daily dose of 1000 µg, per kilogram of prawn's weight. The supply of the feeds continued for 7 days. Group 6 received a LPS-free feed. The blood was collected from the thorax recess of prawns using a syringe holding a KHE culture medium having EDTA on day 0, day 1, day 5 and day 7 after supply of feeds. The collected blood was centrifuged to obtain hemocyte cells. The obtained cells were suspended in a Ca-Mg Hepes culture medium to a concentration of 1×10^6 cells/ml. The cells were crushed by freeze resolution and supersonic waves. The supernatant was separated off by centrifugation and was filtered with a membrane filter. The obtained filtrate (900 µl) was mixed with 100 µl of L-DOPA solution as a substrate solution. Thereafter the mixture was reacted at a temperature of 60°C for 60 minutes. Then the absorbance at 490 nm was measured by a spectrophotometer to assess a phenol oxidase activity (PO activity).

Test results: The biophylaxis of crustaceans involves a cell factor and a liquid factor. The PO activity in hemocyte is deeply concerned with the latter. Thus, it is clarified by assessment of PO activity whether the defensive mechanism of prawns is activated. The PO activity of prawns was determined on day 0, day 1, day 5 and day 7 after supply of feeds for the groups receiving the low molecular weight LPSs (present invention) and th groups rec iving high molecular weight LPSs. The results were tabulated in Table 4.

Table 4

Group	PO activity (absorbance • 490nm)			
	0	1 day	5 days	7 days
Group 1 low MW LPS 20 μg/kg	0.092	0.105	0.199 *1	0.405 *2
Group 2 low MW LPS 40 μg/kg	0.092	0.115	0.201 *1	0.325 *2
Group 3 low MW LPS 100 μg/kg	0.092	0.166 *1	0.170 *1	0.292 *2
Group 4 high MW LPS 100 μg/kg	0.092	0.093	0.124	0.138
Group 5 high MW LPS 1000 μg/kg	0.092	0.104	0.197 *1	0.230 *1
Group 6 feed free of LPS	0.092	0.093	0.136	0.123

*1: significant difference between this group and Group 6 ($P<0.05$)
*2: significant difference between this group and Group 6 ($P<0.01$)

[0045] As apparent from Table 4, the groups receiving the low molecular weight LPSs (present invention) indicated a higher PO activity than Group 6 and a significant difference in this activity from Group 6 ($P<0.01$, $P<0.05$). The group receiving 100 μg of conventional high molecular weight LPS did not increase in PO activity in hemocyte of prawns up to 7 days. The group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher PO activity in hemocyte of prawns($P<0.05$) than Group 6 after 5 and 7 days. The above data show that the low molecular weight LPSs of the present invention can activate the defensive mechanism such as PO activity in hemocyte of prawns even when used in an extremely smaller amount than the high molecular weight LPSs.

<u>Example 5</u> (Effect of preventing acute viremia in kuruma prawns)

[0046] Kuruma prawns having an average weight of 14 g were divided into 7 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively per kilogram of prawn's weight. Group 4 received a high molecular weight LPS as admixed with a feed at a daily dose of 1000 μg, per kilogram of prawn's weight. Group 5 received peptidoglycan (PG) derived from Bifidobacterium thermophilum (Patent No.2547371) as admixed with a feed at a daily dose of 0.2mg (200 μg), per kilogram of prawn's weight. Group 6 received β-1,3-glucan (1,3-G) derived from Schizophyllum commune (JP-B-6-65649) as admixed with a feed at a daily dose of 50mg (50000 μg), per kilogram of prawn's weight. The supply of feeds continued for 18 days. Group 7 (control group) was given a LPS-free feed.

[0047] On day 8 after the start of supply of LPS, infection test was conducted using PRDV (penaeid rod-shaped DNA virus) as a pathogen inducing acute viremia in prawns. Carapaces were removed from the cephalothorax of three prawns which died of acute viremia. The intestine of prawns was crushed and homogenized in 40 ml of sterile seawater. The supernatant (10 ml) was separated off by centrifugation (10,000 × g, 10 minutes, 4°C) and added to 20 liters of seawater. On day 8 after the start of supply of LPS, prawns were infected with acute viremia by immersion in the supernatant for 2 hours. The life or death of prawns was observed for 10 days after infection. The dead prawns were pathologically tested and examined by PCR (polymerase chain reaction) method to confirm whether the prawns died of infection with PRDV.

Test results: Tables 5 and 6 show the total number of dead prawns and a mortality after infection with PRDV in the groups receiving low molecular weight LPSs of the present invention, the group receiving a high molecular weight LPS and the group receiving a LPS-free feed.

Table 5

Group	Days after infection				
	1	2	3	4	5
Group 1 low MW LPS 20 µg/kg	0	0	0	2*	3
Group 2 low MW LPS 40 µg/kg	0	0	3	4	4
Group 3 low MW LPS 100 µg/kg	1	1	3	3	4
Group 4 high MW LPS 100 µg/kg	1	1	6	6	6
Group 5 PG 0.2mg/kg	0	0	2	5	5
Group 6 1,3-G 50mg/kg	0	3	5	7	10
Group 7 feed free of LPS	2	4	13	14	15

* The number indicates the total number of dead prawns. (Other numbers show the same.)

Table 6

Group	Days after infection					Mortality
	6	7	8	9	10	
Group 1 low MW LPS 20 µg/kg	3	3	4	4	4	20 ***
Group 2 low MW LPS 40 µg/kg	6	6	6	7	7	35 ***
Group 3 low MW LPS 100 µg/kg	5	6	8	8	8	40 ***
Group 4 high MW LPS 1000 µg/kg	9	9	10	11	11	55 **
Group 5 PG 0.2mg/kg	7	8	8	8	10	50 **
Group 6 1,3-G 50mg/kg	10	11	11	12	12	60 **
Group 7 feed free of LPS	18	18	19	20	20	100

** : significant difference between this group and Group 7 ($P<0.05$)
*** : significant difference between this group and Group 7 ($P<0.01$)

[0048] All (100%) of prawns died in the control group receiving a LPS-free feed up to 9 days after infection with PRDV. On the other hand, 20%, 35% and 40% of prawns died in the groups receiving 20, 40 and 100 µg, respectively of low molecular weight LPS (present invention). In other words, a low mortality resulted from these groups, and a significant difference ($P<0.01$) exists between these groups and the control group. In contrast, 55% of prawns died in the group receiving 1000 µg of high molecular weight LPS, which means that more prawns died in this group than the groups receiving the low molecular weight LPSs. The above data demonstrate that the low molecular weight LPSs of the present invention can prevent viral infection of prawns and that the low molecular weight LPSs are more efficacious than conventional high molecular weight LPSs.

Example 6 (Activity of activating immune function in fishes)

[0049] Yellowtails weighing 230 g on an average were divided into 6 groups of each 20 yellowtails. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with moist pellets at a daily dose of 20, 40 and 100 µg, respectively per kilogram of yellowtail's weight. Group 4 received a high molecular weight LPS as admixed with moist pellets at a daily dose of 100 µg, and Group 5 received a high molecular weight LPS as admixed with moist pellets at a daily dose of 1000 µg, per kilogram of yellowtail's weight. The feeds were given for 7 days. Group 6 received LPS-free moist pellets. On day 0, day 1, day 5 and day 7 after supply of feeds, a head kidney was xcised from 5 yellowtails. Then hemocyte cells were separated in a plastic petri dish holding a 0.25% NaCl-containng RPMI-

1640-HAH culture medium. Th cells wer pass d through a cell filter to give a cell suspension. The susp nsion was placed over a discontinuous Percoll density gradient. Th reafter a leukocyt layer was formed by centrifugation (1600 rpm., at 4°C for 20 minutes).

[0050] Th leukocyte layer was collected and was subjected to centrifugal washing after which the cells wer suspended in a 10% FBS (fetal bovine serum)-containing 0.25% NaCl-including RPMI-1640-H cultur medium. The number of leukocyte cells in the suspension was adjusted to 1 X 10^6 cells/ml. The leukocyte suspension (500 µl) and 500 µl of a suspension (1 × 10^8 cells/ml) of yeast opsonized with serum of yellowtail were placed into a silicone-treated glass test tube and were incubated at 25°C for 60 minutes with stirring every 10 minutes. After incubation, 5 smears per yellowtail were produced, subjected to Wright's staining and enclosed with Eukitt. The hemocyte cells (200 cells per smear) were observed at random under an optical microscope. Then the number of yeast cells phagocytized into leukocyte was counted. The phagocytosis index was given by the same equation as in Example 3. The results are shown in Tables 7 and 8.

Table 7

Group	Phagocytosis index of leukocyte	
	0	1 day
Group 1 low MW LPS 20 µg/kg	7.3±2.30	12.7±2.65 *1
Group 2 low MW LPS 40 µg/kg	7.3±2.30	17.9±3.99 *2
Group 3 low MW LPS 100 µg/kg	7.3±2.30	18.6±4.12 *2
Group 4 high MW LPS 100 µg/kg	7.3±2.30	6.3±2.24
Group 5 high MW LPS 1000 µg/kg	7.3±2.30	8.2±2.18
Group 6 feed free of LPS	7.3±2.30	6.6±1.19

*1: significant difference between this group and Group 6 (P<0.05)
*2: significant difference between this group and Group 6 (P<0.01)

Table 8

Group	Phagocytosis index of leukocyte	
	5 days	7 days
Group 1 low MW LPS 20 µg/kg	39.2±2.54 *2	52.7±4.08 *2
Group 2 low MW LPS 40 µg/kg	37.4±4.28 *2	37.0±3.11 *2
Group 3 low MW LPS 100 µg/kg	42.6±5.35 *2	36.5±4.32 *1
Group 4 high MW LPS 100 µg/kg	11.2±3.05	10.6±2.96
Group 5 high MW LPS 1000 µg/kg	22.7±3.16 *1	31.8±3.52 *1
Group 6 feed free of LPS	9.0±2.04	7.7±1.73

*1: significant difference between this group and Group 6 (P<0.05)
**2: significant difference between this group and Group 6 (P<0.01)

[0051] As apparent from Tables 7 and 8, any groups of yellowtails receiving the low molecular weight LPSs (present invention) indicated a higher phagocytosis index in leukocyte of yellowtails than Group 6 and a significant difference (P<0.01, P<0.05) in this index from Group 6. However, the group receiving 100 µg of conventional high molecular weight LPS did not increase the phagocytosis index in leukocyte of yellowtails after 7 days. The group receiving 1000 µg of conventional high molecular weight LPS showed a significantly higher phagocytosis index (P<0.01) in leukocyte of yellowtails than Group 6 after 5 days. The above data show that the low molecular weight LPSs of the present invention can activate the immune system of fishes such as phagocytosis in leukocyte in an extremely smaller amount than

conventional high molecular weight LPSs.

Example 7 (Effect of preventing enterococcus disease in yellowtails)

[0052] Yellowtails weighing 63 g on an average were divided into 5 groups of each 30 yellowtails. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with moist pellets at a daily dose of 20, 40 and 100 µg, respectively per kilogram of yellowtail's weight. Group 4 received a high molecular weight LPS as admixed with moist pellets at a daily dose of 1000 µg per kilogram of yellowtail's weight. Group 5 (control) received LPS-free moist pellets. On day 7 after supply of feeds, the yellowtails were intraabdominally inoculated with Enterococcus Seriolicida as a pathogen causing enterococcus disease of yellowtail in an amount of 4.0 X 10^6 cells per yellowtail. A mortality 15 days after inoculation was determined. The results are shown in Tables 9 and 10.

Table 9

Group	Days after infection								
	1	2	3	4	5	6	7	8	9
Group 1 low MW LPS 20 µg/kg	0	0	0	0	0	0	0	0	1*
Group 2 low MW LPS 40 µg/kg	0	0	0	1	1	2	2	4	4
Group 3 low MW LPS 100 µg/kg	0	0	0	0	0	1	3	3	5
Group 4 high MW LPS 1000 µg/kg	0	0	0	1	1	1	3	3	3
Group 5 feed free of LPS	0	0	1	2	7	7	10	12	16

* The number indicates the total number of dead yellowtails. (Other numbers show the same.)

Table 10

Group	Days after infection						Mortality (%)
	10	11	12	13	14	15	
Group 1 low MW LPS 20 µg/kg	3	3	3	3	4	4	13.3 ***
Group 2 low MW LPS 40 µg/kg	7	8	8	8	8	8	26.7 **
Group 3 low MW LPS 100 µg/kg	5	5	5	7	7	7	23.3 **
Group 4 high MW LPS 1000 µg/kg	5	9	10	10	11	11	36.7 **
Group 5 feed free of LPS	16	16	17	22	22	22	73.3

** : significant difference between this group and Group 5 ($P<0.05$)
***: significant difference between this group and Group 5 ($P<0.01$)

[0053] On 15th day after inoculation of E. Seriolicida, 73.3% of yellowtails died in the control group receiving LPS-free feed. In contrast, a low mortality is indicated by the groups receiving the low molecular weight LPSs of the present invention, i.e. 13.3% from the group receiving 20 µg, 26.7% from the group receiving 40 µg and 23.3% from the group receiving 100 µg. In other words, there is a significant difference ($P<0.05$) in mortality between these groups and the control group. On the other hand, a mortality of 36.7% resulted from the group receiving 1000 µg of high molecular weight LPS. This group showed a higher mortality than the groups receiving low molecular weight LPSs. The above results show that the low molecular weight LPSs of the present invention can protect fishes against viral infection and are more efficacious than conventional high molecular weight LPSs.

INDUSTRIAL APPLICABILITY

[0054] According to the present invention, there is provided a safe feedstuff additive for growing crustaceans and fishes, the feedstuff additive being capable of preventing infectious diseases by properly activating their intrinsic

immune function even when used in a small amount, being capable of preventing the perish of crustac ans and fishes, and b ing free from the problems of public h alth hazards such as the feedstuff additive remaining in crustaceans and fishes.

Claims

1. A feedstuff additive for crustaceans or fishes, characterized in that it is prepared from gram-negative bacteria, that it has a molecular weight of 5000 ± 2000 as measured by SDS-PAGE method using a protein marker, that it is substantially free of high molecular weight lipopolysaccharide, that it contains a low molecular weight lipopolysaccharide as an effective component and that it is capable of activating immunity or preventing infection in crustaceans or fishes.

2. A feedstuff additive for crustaceans or fishes comprising the low molecular weight lipopolysaccharide of claim 1 and a carrier acceptable for crustaceans and fishes.

3. Use of the low molecular weight lipopolysaccharide of claim 1 for the preparation of a feedstuff additive for crustaceans or fishes.

4. A method of activating immunity or preventing infection in crustaceans or fishes comprising administering an effective amount of the low molecular weight lipopolysaccharide of claim 1 to crustaceans or fishes.

5. An agent for preventing the perish of crustaceans or fishes comprising the low molecular weight lipopolysaccharide of claim 1 as an effective component.

6. An agent for preventing the perish of crustaceans or fishes comprising the low molecular weight lipopolysaccharide of claim 1 and a carrier acceptable for crustaceans and fishes.

7. Use of the low molecular weight lipopolysaccharide of claim 1 for the preparation of an agent for preventing the perish of crustaceans or fishes.

8. A method of preventing the perish of crustaceans or fishes comprising administering an effective amount of the low molecular weight lipopolysaccharide of claim 1 to crustaceans or fishes.

9. A feedstuff additive according to claim 1, wherein the gram-negative bacteria are those pertaining to genus Pantoea.

10. A feedstuff additive according to claim 9, wherein the gram-negative bacteria are Pantoea agglomerans.

11. A feed for crustaceans or fishes comprising the feedstuff additive of claim 1.

12. A feed for crustaceans or fishes comprising the agent for preventing the perish of claim 5.

13. A method of breeding crustaceans or fishes comprising administering a feed of claim 11 to crustaceans or fishes.

14. A method of breeding crustaceans or fishes comprising administering a feed of claim 12 to crustaceans or fishes.

15. A feedstuff additive according to claim 1, wherein the infectious diseases is acute viremia of crustaceans, their vivrio diseases, parasitosis or mycosis ; iridovirus infectious diseases of fishes, their rhabdovirus diseases, neuronecrosis, infectious hemopoietic organ necrosis, pseudotuberculosis, streptococcic diseases, enterococcus diseases, vivrio diseases, cold-water disease, Pseudomonas diseases, gliding-bacteria diseases or Saprolegnia diseases.

16. A feedstuff additive according to claim 1, wherein the high molecular weight lipopolysaccharide is one having a molecular weight of at least 8,000.

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添付公開書類
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(54)Title: ADDITIVES FOR CRUSTACEAN OR FISH FEEDS AND FEEDS

(54)発明の名称 甲殻類又は魚類用飼料添加剤及び飼料

(57) Abstract

Additives for crustacean or fish feeds having an immunopotentiating and infection-preventing effects, characterized by containing as the active ingredient a low-molecular weight lipopolysaccharide which is obtained from gram-negative microbial cells, has a molecular weight of 5000±2000 when measured by the SDS-PAGE method with the use of protein markers, and is substantially free from high-molecular weight lipopolysaccharides; and crustacean or fish feeds characterized by containing these additives.

(57)要約

グラム陰性の微生物菌体から得られ、タンパク質マーカーを用いてＳＤＳ－ＰＡＧＥ法で測定した分子量が５０００±２０００で、高分子量リポポリサッカライドを実質的に含まない、低分子量リポポリサッカライドを有効成分として含有することを特徴とする免疫賦活、感染症予防効果を有する甲殻類又は魚類用飼料添加剤、及びこれを添加したことを特徴とする甲殻類又は魚類用飼料。

明 細 書

甲殻類又は魚類用飼料添加剤及び飼料

技術分野

本発明は、甲殻類又は魚類の飼料添加剤及び該飼料添加剤を添加した飼料に係わり、特に免疫賦活、感染症予防に著効を示す飼料添加剤と、この飼料添加剤を適宜の割合で添加した飼料、並びにこれらを用いた予防方法及び飼育方法に関する。

背景技術

近年、甲殻類や魚類の養殖産業が発展するに伴って、細菌病並びにウィルス病が多発し、甚大な経済的被害をもたらしている。甲殻類や魚類の疾病については、クルマエビの急性ウィルス血症、ビブリオ病、ブリの類結節症、腸球菌症、アユの冷水病、シュードモナス病、マダイ、カンパチ、ブリなどのイリドウィルス感染症などの発生が多く、経済的被害が大きい。

これらの病気のうち細菌性疾病については、治療薬として抗生物質や合成抗菌剤が用いられているが、抗菌性物質に対する耐性菌が出現し、充分な治療効果が得られていない。また、使用した薬剤の甲殻類、魚類への残留による公衆衛生上の問題が生じていることから、化学療法に依存しない予防対策が強く望まれている。一方、甲殻類及び魚類のウィルス病については、ワクチンや治療薬が開発されておらず、病気が依然として多発している状況にある。

甲殻類及び魚類の免疫機能の増強と感染症の予防を目的として、ビィフィドバクテリウム・サーモフィラム由来のペプチドグリカン（特許第２５４７３７１号公報）、バチラス属などグラム陽性菌の細胞壁成分（特公平３－１７３８２６号

公報）、スエヒロタケ由来のβ－１，３－グルカン（特公平６－６５６４９号公報）などの多糖類を利用することは、既に知られている。また、高分子量リポポリサッカライドが魚類の免疫機能を活性化することは、既に報告されている（Salati, F. and R. Kusuda、日本水産学会誌、５３巻、２０１～２０４ページ、１９８７年）（Odean, M. J. ら、Infection and Immunity、５８巻、４２７～４３２ページ、１９９０年）。

本発明で使用される低分子量リポポリサッカライド（以下、低分子量ＬＰＳと略す）は、上記のグラム陽性菌由来のペプチドグリカン、細胞壁成分及びキノコ由来のβ－１，３－グルカンと基本構造及び成分が異なり、特異な脂質リピドＡとそれに共有結合したＲコアと呼ばれるオリゴ糖、さらにＯ特異多糖の３成分よりなる物質であり、腫瘍壊死因子（ＴＮＦ）産生効果増強に基づく動物用の免疫機能活性剤として公知であるが、甲殻類及び魚類の感染症予防作用については全く知られていなかった。また、既報の研究に用いられた高分子量リポポリサッカライド（ＬＰＳ）は分子量が１００万～１０００万と、極めて大きく毒性が強いために甲殻類及び魚類に長期間投与して、常時免疫機能を活性化しておくことが不可能であった。さらに、前述の既知の物質は分子量が大きく腸管からの吸収が悪いために、多量の経口投与を必要とし、長期間投与すると免疫機能が低下するものが多かった。

既に述べた様に、甲殻類及び魚類には種々の感染症が多発し、その結果斃死に至るものもあり、甚大な経済的被害をもたらしている。この背景には、これらの甲殻類又は魚類が限られた環境で過密に飼育されることによる免疫機能の低下が挙げられる。また、低下した免疫機能を活性化する目的で、既に種々の物質が用いられているが、甲殻類は抗体を産生する能力がなく、脊椎動物にみられるリンパ球、好中球、好塩基球等が認められない等、また魚類は抗体を産生する能力が限定されており、更に変温動物であるために抗体産生が水温に大きく影響され、

このような免疫機能は充分機能していない等、両者は哺乳動物とは生体防御機構にかなりの差があること（Ｆｉｓｈ Ｐａｔｈｏｌｏｇｙ, ３０（２），１４１－１５０，１９９５．６）、既往のＬＰＳのように毒性が強いために養殖現場では使用できない場合があること、長期間投与するとむしろ免疫機能が低下するものが多いこと等により、甲殻類及び魚類の感染症防止に満足できるものはなかった。

本発明の目的は甲殻類及び魚類が本来的に備えている免疫機能を、ごく微量で的確に活性化して感染症を予防し、薬物残留などの公衆衛生上の問題のない、安全な甲殻類及び魚類を育成するための飼料添加剤、この飼料添加剤を添加した飼料、並びにこれらを用いた予防方法及び飼育方法を提供することにある。

発明の開示

本発明はグラム陰性の微生物菌体から得られ、タンパク質マーカーを用いてＳＤＳ－ＰＡＧＥ法で測定した分子量が５０００±２０００で、高分子量リポポリサッカライドを実質的に含まない、低分子量リポポリサッカライドを有効成分として含有することを特徴とする免疫賦活、感染症予防効果を有する甲殻類又は魚類用飼料添加剤に係る。

また本発明は、上記低分子量リポポリサッカライド及び甲殻類及び魚類に許容される担体を含有する甲殻類又は魚類用飼料添加剤に係る。

また本発明は、甲殻類又は魚類用飼料添加剤を製造するための上記低分子量リポポリサッカライドの使用に係る。

また本発明は、上記低分子量リポポリサッカライドの有効量を甲殻類又は魚類に投与することを特徴とする甲殻類又は魚類の免疫賦活、感染症予防方法に係る。

また本発明は、上記低分子量リポポリサッカライドを有効成分として含有することを特徴とする甲殻類又は魚類の斃死予防剤に係る。

また本発明は、上記低分子量リポポリサッカライド及び薬学的に許容される担

体を含有する甲殻類又は魚類の斃死予防剤に係る。

また本発明は、甲殻類又は魚類の斃死予防剤を製造するための上記低分子量リポポリサッカライドの使用に係る。

また本発明は、上記低分子量リポポリサッカライドの有効量を甲殻類又は魚類に給餌することを特徴とする甲殻類又は魚類の斃死予防方法に係る。

また本発明は、グラム陰性の微生物菌体がパントエア属に属する微生物菌体である甲殻類又は魚類用飼料添加剤に係る。

また本発明は、グラム陰性の微生物菌体がパントエア・アグロメランスである甲殻類又は魚類用飼料添加剤に係る。

また本発明は、上記飼料添加剤又は斃死予防剤を添加したことを特徴とする甲殻類又は魚類用飼料に係る。

また本発明は上記飼料を甲殻類又は魚類に与えることを特徴とする甲殻類又は魚類の飼育方法に係る。

本発明は、グラム陰性の微生物菌体から例えば特開平８－１９８９０２号公報において開示された方法によって精製した物質で、分子量が５０００±２０００の低分子量ＬＰＳを飼料に添加して、甲殻類及び魚類に投与したところ、甲殻類・魚類が本来的に備えている免疫機能が活性化され、ウィルスや細菌による感染症が防御され、斃死が予防され、しかも安全性が非常に高いことを確認して、本発明を完成した。

本発明の低分子量ＬＰＳは、前記したようにグラム陰性の微生物菌体から、例えば特開平８－１９８９０２号公報において開示された方法によって得られた分子量が５０００±２０００のリポポリサッカライドを言うが、その特徴は従来の高分子量ＬＰＳ（分子量１００万～１０００万）に比べて甲殻類及び魚類に対する安全性が高く、顕著な免疫活性化作用及び感染症予防効果並びに斃死予防効果を有することにある。

本発明において、実質的に高分子量リポポリサッカライドを含まないとは８０００以上の分子量のものを含まないことを意味する。

本発明において、グラム陰性の微生物菌体としては例えばパントエア属、サルモネラ属、アエロモナス属、セラチア属、エンテロバクロー属等に属する微生物菌体、その他特開平４－９９４８１号公報に記載のグラム陰性の微生物菌体などを挙げることができる。好ましい微生物はパントエア属に属する微生物であり、より好ましくはパントエア・アグロメランス（Pantoea agglomerans）である。

この発明の低分子量ＬＰＳは、グラム陰性の微生物等を、常法により培養し、培地から菌体を集め、集めた菌体から公知の方法、例えば、熱フェノール法［オー・ウエストファール（O. Westphal）編、メソッズ・イン・カーボハイドレート・ケミストリー(Methods in Carbohydrate Chemistry)、第５巻、第８３ページ、アカデミック・プレス(Academic Press)、１９６５年］、により抽出し、さらに、陰イオン交換樹脂により精製して製造できる。すなわち、微生物の菌体を蒸留水に懸濁し、この懸濁液を蒸留水および等容量の熱フェノールの混合液に添加して撹拌し、次いで遠心分離して水層を回収し、この水層を透析してフェノールを除去し、限外濾過法により濃縮して粗ＬＰＳ画分を採取し、この画分を常法の陰イオン交換クロマトグラフィー（例えば、モノＱ－セファロースまたはＱ－セファロースを使用する）により精製し、常法により脱塩する。

このようにして得られた精製ＬＰＳは特開平４－１８７６４０号公報、特開平４－４９２４０号公報、特開平４－９９４８１号公報および特開平５－１５５７７８号公報に開示される分子量５，０００から６，０００程度のＬＰＳと実質的に等しい。さらに、得られた精製、ＬＰＳを、例えばデオキシコール酸ナトリウム等の界面活性剤の存在下でゲル濾過し、低分子量ＬＰＳを含有する画分のみを回収し、混在する高分子量ＬＰＳを除去することによって、高度に精製された本発明の低分子量ＬＰＳを得ることができる。この界面活性剤存在下でのゲル濾過の

工程は、特開平４－１８７６４０号公報、特開平４－４９２４０号公報および特開平５－１５５７７８号公報に開示される分子量５，０００から６，０００程度のＬＰＳを更に高度に精製するためのものであり、この工程により混在する高分子量ＬＰＳが完全に排除されるのである。

本発明の対象となる甲殻類とは、クルマエビ（Penaeus japonicus）、ウシエビ（Penaeus monodon）、コウライエビ（Penaeus chinensis）、バナナエビ（Penaeus morguiensis）等のクルマエビ類を含む全てのエビ類（lobster, shrimp, prawn を含む）、上海ガニ、ガザミ等の全てのカニ類を含み、好ましくはエビ類であり、更に好ましくはクルマエビ類である。魚類とは、ブリ、フグ、マダイ、ヒラメ、ウナギ、ニジマス等の、全ての魚類を含む。感染症とは甲殻類の急性ウィルス血症、ビブリオ病、Bpistylis sp., Zoothamnium sp. 等の寄生症、あるいはLagenidium sp., Siropidium sp. 等の真菌症、魚類のイリドウィルス感染症、ラブドウィルス病、神経壊死症、伝染性造血器壊死症、類結節症、連鎖球菌症、腸球菌症、ビブリオ病、冷水病、シュードモナス病、滑走細菌症、水カビ病のほか、全てのウィルス、マイコプラズマ、細菌、真菌及び寄生虫に起因する感染症を言い、好ましくは、甲殻類の急性ウイルス血症、魚類の連鎖球菌症、腸球菌症、ビブリオ症である。

本発明においては、低分子量ＬＰＳを、そのまま、あるいは公知の担体、安定剤等を加えて、更に必要に応じてビタミン、アミノ酸類、ミネラル等の各種養分、抗酸化剤、抗生物質、抗菌剤及びその他の添加剤等を加えて、甲殻類又は魚類用の飼料添加剤となしてもよく、その形状としては、粉体、顆粒、ペレット、懸濁液等の適宜の状態に調製すればよい。本発明の飼料添加剤を給餌する場合は、甲殻類又は魚類に対し、単独で給餌しても良いし、飼料に混合して給餌しても良い。給餌時期は、疾患予防のために常時給餌しても、また飼養後半に添加してもよい。

また、本発明の飼料は特に限定されるものではなく、粉末飼料、固型飼料、モ

イストペレット飼料、ドライペレット飼料、ＥＰ（Extruder　Pellet）飼料、生餌など、どのような飼料でもよい。

本発明において低分子量ＬＰＳの飼料添加剤又は飼料等への配合量は広い範囲から選択できるが、好ましくは飼料添加剤又は飼料に対して０．０００００１～０．００１重量％、特に好ましくは０．００００２～０．０００５重量％であるが、これに限定されるものではない。低分子量ＬＰＳの給餌量は適宜決定すれば良いが、例えば甲殻類及び魚類の体重１ｋｇあたり１日量として１～１００μｇ、好ましくは１０～２０μｇを投与するのがよいが、これに限定されるものではない。

発明を実施するための最良の形態

以下に実施例を挙げて本発明について説明するが、これら実施例に何ら限定されるものではない。尚、実施例に用いられた低分子量ＬＰＳとは分子量約５，０００のＬＰＳであり、高分子量ＬＰＳとは分子量８，０００～５万のＬＰＳである。

参考例１　（低分子量ＬＰＳの製造）

トリプトン（ディフコ社製）１０ｇ、酵母エキス（ディフコ社製）５ｇ、NaCl（和光純薬工業社製、特級）１０ｇを蒸留水１リットルに添加し、NaOHでｐＨを７．５に調整し、オートクレーブで滅菌し、別に滅菌したグルコース（和光純薬工業社製、特級）を０．１％の割合で添加した培地（以下Ｌ－肉汁培地と記載する）１００ｍｌの入った５００ｍｌ容の坂口フラスコに、－８０℃で保存されているパントエア・アグロメランス（Pantoea agglomerans）保存菌株から単一コロニーを分離して接種し、３５℃で１夜振とう培養し、そのまま全量を１，０００ｍｌのＬ－肉汁培地の入った３リットル容の坂口フラスコに接種し、同様に培養した。

さらに、７リットルのＬ－肉汁培地の入った１０リットル容の卓上型ファーメンター（丸菱バイオエンジ社製）に培養した菌体を接種し、同条件で通気培養し、のち集菌し、約７０ｇの湿菌体を回収し、これを凍結保存した。凍結保存菌体約＋＋７０ｇを５００ｍｌの蒸留水に懸濁し、５００ｍｌの９０％熱フェノールを添加して６５～７０℃で２０分間撹拌し、冷却し、１０，０００Ｇ、４℃で２０分間遠心処理し、水層を回収した。フェノール層を更に１回前記と同一の操作を反復し、回収した２回の水層を合し、１夜透析してフェノールを除去し、透析内液を限外濾過装置（アドヴァンテック・トーヨー社製、ＵＫ－２００）を用いて分子量２０万カットーオフ膜により２気圧の窒素ガス下で限外濾過濃縮した。

得られた粗ＬＰＳ凍結乾燥物を蒸留水に溶解し、フィルター滅菌し、緩衝液を添加し、陰イオン交換クロマトグラフィー（ファルマシア社製、Ｑ－セファロース・ファースト・フロー）にかけ、１０mMトリス－ＨＣl（ｐＨ７．５）および１０mMのNaClを含む緩衝液で試料溶液をカラムに通液し、２００～４００ｍMNaCl／１０mMトリス－ＨＣl（ｐＨ７．５）でリムラス活性画分を溶出させた。この溶出液を前記と同一条件で限外濾過して脱塩および濃縮し、凍結乾燥し、約７０ｇの湿菌体から約３００ｍｇの精製ＬＰＳを得た。

得られた精製ＬＰＳ１００ｍｇを５ｍｇ／ｍｌの濃度で可溶化緩衝液［３％デオキシコール酸ナトリウム（和光純薬社製）０．２Ｍ塩化ナトリウム、５mMEDTA－２Ｎａおよび２０mMトリス－塩酸からなり、ｐＨ８．３］に溶解し、精製ＬＰＳ溶液２０ｍｌをセファクリルＳ－２００ＨＲカラム（ファルマシア社製）の上部に静かに重層し、溶出緩衝液［０．２５％デオキシコール酸ナトリウム（和光純薬社製）、０．２Ｍ塩化ナトリウム、５mMEDTAおよび１０mMトリス－塩酸からなり、ｐＨ８．３］により流速１６ｍｌ／時で８００ｍｌ（５０時間）溶出した。

ペリスタポンプＰＩ（ファルマシア社製）を用いて流速を制御しながら、得ら

れた溶出液を、フラクションコレクター（アドバンテック社製、ＳＦ２１２０）により分画し、最初の２４０ｍｌ（２４フラクション分）を廃棄し、その後１０ｍｌ／フラクションで８０フラクションまで分画した。溶出した各画分について原液または希釈液でフェノール／硫酸法（福井作蔵、「還元糖の定量法・第２版」、第５０～５２ページ、学会出版センター、１９９０年）により糖の定量を行い、溶出状態を調べた。得られた溶出状態の結果から、ＬＰＳの存在が予想される分画（フラクション３０～６０）のうち、フラクション３７～５５の各フラクション０.５ｍｌを用いてＳＤＳ－ＰＡＧＥを行い、ＬＰＳの分画パターンを調べた。

その結果、フラクション４５－５５は低分子量（分子量約５,０００）ＬＰＳのみが認められ、フラクション３７－４４は高分子量および低分子量の両方のＬＰＳが認められたので、フラクション４５－５５の低分子量ＬＰＳ分画を次のとおりさらに精製した。

各画分を混合して凍結乾燥し、エタノールに懸濁し、遠心分離によりエタノールに可溶なデオキシコール酸を除去し、低分子量ＬＰＳを不溶性画分に回収した。低分子量ＬＰＳ画分のエタノール処理をさらに２回反復し、デオキシコール酸を除去し、次に７０％エタノールに再度懸濁し、遠心分離で緩衝液成分を除去し、この操作をさらに３回反復し、低分子量ＬＰＳを不溶性画分に回収し、凍結乾燥し、精製した低分子量ＬＰＳを約２０ｍｇ得た。

実施例１　　（甲殻類に対する低分子量ＬＰＳの安全性）

平均体重２０ｇのクルマエビを２０尾ずつの５群に分け、本発明の低分子量ＬＰＳ（分子量約５,０００）をエビの体重１ｋｇたり１区には５０ｍｇ、２区には１００ｍｇを、また従来の高分子量ＬＰＳ（大腸菌由来ＬＰＳ、Ｅ. coli Ｏ１１１，ＤＩＦＣＯ社製、分子量約８,０００～５万）を３区には１０ｍｇ、４区には２０ｍｇとなるように第３腹節筋肉内に投与した。５区にはＬＰＳを含ま

ない生理食塩水を投与した。投与後１２０時間までのエビの生死を確認し、斃死率を求めた。結果を表１に示した。

【表１】

試　験　区　分	斃死尾数／供試尾数	斃死率（%）
１区低分子量ＬＰＳ５０ｍｇ／ｋｇ	０／２０	０
２区低分子量ＬＰＳ１００ｍｇ／ｋｇ	０／２０	０
３区高分子量ＬＰＳ１０ｍｇ／ｋｇ	１３／２０	６５
４区高分子量ＬＰＳ２０ｍｇ／ｋｇ	２０／２０	１００
５区生理食塩水区	０／２０	０

表１から明らかなように、高分子量ＬＰＳの１０ｍｇ及び２０ｍｇ区の斃死率がそれぞれ６５、１００％であったのに対して、低分子量ＬＰＳの５０ｍｇ区及び１００ｍｇ区は、いずれも斃死する個体が全くみられなかった。このことから、本発明の低分子量ＬＰＳは従来の高分子量ＬＰＳに比べて、エビに対する安全性が極めて高い物質であることが明らかである。

実施例２　（魚類に対する低分子量ＬＰＳの安全性）

平均体重８５ｇのマゴイを４０尾ずつ３群に分け、マゴイの体重１ｋｇ当たり、１区には低分子量ＬＰＳ１００ｍｇを、２区には高分子量ＬＰＳ（E. coli 0111，ＤＩＦＣＯ社製）２０ｍｇを、いずれも背部筋肉内に投与した。３区にはＬＰＳを含まない生理食塩水を投与した。投与後１２０時間までのマゴイの生死を確認し、斃死率を求めた。結果を表２に示した。

【表２】

試　験　区　分	斃死尾数／供試尾数	斃死率（%）
１区低分子量ＬＰＳ１００ｍｇ／ｋｇ	０／４０	０
２区高分子量ＬＰＳ２０ｍｇ／ｋｇ	３４／４０	８５
３区生理食塩水区	０／４０	０

表２から明らかなように、高分子量ＬＰＳ２０ｍｇ区の斃死率が８５％であったのに対して、低分子量ＬＰＳ１００ｍｇ区は斃死する個体が全くみられなかった。このことから、本発明の低分子量ＬＰＳは従来の高分子量ＬＰＳに比べて、

魚類に対する安全性が極めて高い物質であることが明らかである。

実施例３　（甲殻類における血球の貪食能に対する活性化作用）

平均体重２０ｇのクルマエビを２０尾ずつの６群に分け、本発明区の１、２、３区には低分子量ＬＰＳをエビの体重１ｋｇ当たり１日量として、それぞれ２０、４０、１００μｇを、また高分子量ＬＰＳを４区には１００μｇ、５区には１０００μｇとなるように飼料に混合して７日間投与した。６区にはＬＰＳを含まない飼料を与えた。投与開始０、１、５、７日後に、抗凝固剤としてのＬ－システインを含むＫ－１９９培地を入れた注射器を用いてエビの胸洞から採血し、遠心分離によって血球を得た。懸濁液１μｌ当たり1×10^{5}細胞の血球と1×10^{8}個のラテックスビーズ（直径１．９８６μｍ）を混合し、２５℃で３０分間反応させたのち、グルタールアルデヒドで固定後、風乾した。風乾後、ギムザ液で染色し、オイキットを用いてスライドガラス上に固定した。この標本をエビ１尾当たり５枚作製し、落射蛍光顕微鏡を用いて標本１枚当たり２００個の血球を無作為に観察し、血球のラテックスビーズ貪食率、血球１細胞当たりのビーズ取り込み数を調べ、下式によって貪食指数を求めた。

貪食率＝［ビーズを取り込んだ血球数／観察した血球の総数］×１００

平均取り込み数＝血球に取り込まれたビーズ数／ビーズを取り込んだ血球数

貪食指数＝［ビーズを取り込んだ血球数／観察した血球の総数］×［血球に取り込まれたビーズ数／観察した血球の総数］×１００

試験結果：甲殻類の生体防御機構は、細胞性因子と液性因子によって構成されており、前者には異物に対する血球の貪食能が深く関与していることから、エビの生体防御能が活性化しているか否かは、異物に対する血球の貪食活性を調べることによって明らかになる[高橋幸則ら：魚病研究３０（２）、１４１～１５０（１９９５）]。そこで、本発明の低分子量ＬＰＳ区及び高分子量ＬＰＳ区における投与開始０、１、５、７日後の貪食指数を調べ、表３に示した。

【表３】

試験区分	血球の貪食指数	
	0	1（日後）
1区低分子量LPS20μg/kg	0.9±0.18	2.1±0.61 ※2
2区低分子量LPS40μg/kg	0.9±0.18	3.3±1.16 ※2
3区低分子量LPS100μg/kg	0.9±0.18	3.8±1.00 ※2
4区高分子量LPS100μg/kg	0.9±0.18	0.7±0.31
5区高分子量LPS1000μg/kg	0.9±0.18	1.1±0.63
6区LPS無添加飼料区	0.9±0.18	0.5±0.24

試験区分	血球の貪食指数	
	5	7（日後）
1区低分子量LPS20μg/kg	3.2±0.71 ※2	8.4±1.37 ※2
2区低分子量LPS40μg/kg	4.5±0.75 ※2	3.7±1.02 ※2
3区低分子量LPS100μg/kg	3.1±0.94 ※2	2.8±0.70 ※1
4区高分子量LPS100μg/kg	0.7±0.82	1.2±0.44
5区高分子量LPS1000μg/kg	2.1±0.58 ※1	2.9±0.68 ※1
6区LPS無添加飼料区	0.7±0.5	1.1±0.56

※１：６区との間に有意差（Ｐ＜０．０５）

※２：６区との間に有意差（Ｐ＜０．０１）

表３から明らかなように、低分子量ＬＰＳを投与したエビにおける血球の貪食指数は、いずれの本発明区ともに６区に比べて高く、有意な差が見られた（Ｐ＜０．０１、０．０５）。しかし、従来の高分子量ＬＰＳを１００μｇ投与したエビにおける血球の貪食指数は投与１、５、７日後ともに上昇せず、１０００μｇ区においては投与５、７日後に６区よりも有意に高くなった（Ｐ＜０．０５）。以上のことから、本発明の低分子量ＬＰＳは高分子量ＬＰＳよりも極めて微量で、エビ血球の貪食活性などの生体防御能を活性化することが明らかである。

実施例４　（甲殻類における血球のフェノールオキシダーゼに対する活性化作用）

平均体重２０ｇのクルマエビを２０尾ずつの６群に分け、本発明区の１、２、３区には低分子量ＬＰＳをエビの体重１ｋｇ当たり１日量として、それぞれ２０、

４０、１００μｇを、また高分子量ＬＰＳを４区には１００μｇ、５区には１０００μｇとなるように飼料に混合して７日間投与した。６区にはＬＰＳを含まない飼料を与えた。投与開始０、１、５、７日後に、ＥＤＴＡを含むＫＨＥ培地を入れた注射器を用いてエビの胸洞から採血し、遠心分離によって血球を得た。得られた血球をCa−Mg Hepes培地に１×１０^{6}細胞／ｍｌとなるように懸濁したのち、凍結融解と超音波によって破壊し、遠心分離によって得られた上清をメンブレンフィルターでろ過した。この液９００μｌと基質溶液としてのＬ−ＤＯＰＡ溶液１００μｌを混合後、６０℃温度下で６０分間反応させ、分光光度計を用いて４９０ｎｍにおける吸光度を測定し、フェノールオキシダーゼ（ＰＯ）活性とした。

試験結果：甲殻類の生体防御機構は、細胞性因子と液性因子によって構成されており、後者には血球のＰＯ活性が深く関与していることから、エビの生体防御能が活性化しているか否かは、ＰＯ活性を調べることによっても明らかになる。そこで、本発明の低分子量ＬＰＳ区及び高分子量ＬＰＳ区における投与開始０、１、５、７日後のＰＯ活性を調べ、表４に示した。

【表４】

試験区分	PO活性（吸光度・490nm）			
	0	1	5	7（日後）
1区低分子量 LPS20μg／kg	0.092	0.105	0.199 ※1	0.405 ※2
2区低分子量 LPS40μg／kg	0.092	0.115	0.201 ※1	0.325 ※2
3区低分子量 LPS100μg／kg	0.092	0.166 ※1	0.170 ※1	0.292 ※2
4区高分子量 LPS100μg／kg	0.092	0.093	0.124	0.138
5区高分子量 LPS1000μg／kg	0.092	0.104	0.197 ※1	0.230 ※1
6区LPS無添加飼料区	0.092	0.093	0.136	0.123

※１：６区との間に有意差（Ｐ＜０．０５）

※２：６区との間に有意差（P＜０．０１）

表４から明らかなように、低分子量ＬＰＳを投与したエビにおける血球のＰＯ活性は、いずれの本発明区ともに６区に比べて高く、有意な差がみられた（P＜０．０１、０．０５）。しかし、従来の高分子量ＬＰＳを１００μｇ投与したエビにおける血球のＰＯ活性は投与７日後まで上昇せず、１０００μｇ区においては投与５、７日後に６区よりも有意に高くなった（P＜０．０５）。以上のことから、本発明の低分子量ＬＰＳは高分子量ＬＰＳよりも極めて微量で、エビ血球のＰＯ活性などの生体防御能を活性化することが明らかである。

実施例５　（クルマエビ急性ウィルス血症に対する予防効果）

平均体重１４ｇのクルマエビを２０尾ずつの５群に分け、本発明区の１、２、３区には低分子量ＬＰＳをエビの体重１ｋｇ当たり１日量として、それぞれ２０、４０、１００μｇを、また４区には高分子量ＬＰＳを１０００μｇとなるように飼料に混合して１８日間投与した。５区には特許第２５４７３７１号公報記載のビィフィドバクテリウム・サーモフィラム由来のペプチドグリカン（ＰＧ）を０．２ｍｇ／ｋｇ（２００μｇ／ｋｇ）となるように、６区には特公平６－６５６４９号公報記載のスエヒロタケ由来のβ－１，３－グルカン（１，３－Ｇ）を５０ｍｇ／ｋｇ（５００００μｇ／ｋｇ）となるように飼料に混合して１８日間投与した。７区の対照区には、ＬＰＳを含まない飼料を与えた。

ＬＰＳを投与開始８日後に、クルマエビ急性ウィルス血症の原因ウィルスであるＰＲＤＶ（penaeid rod-shaped ＤＮＡ virus）を用いて感染試験を行った。感染方法は、本病によって斃死した３尾のクルマエビの頭胸部甲皮を剥がしたのち、４０ｍｌの滅菌海水中でホモジナイズし、遠心分離（１０，０００×ｇ、１０分間、４℃）によって得られた上清１０ｍｌを２０リットルの海水に加えた。この中にＬＰＳを投与開始８日後のエビを２時間浸漬する方法によって感染させた。感染後１０日間の斃死状況を観察し、斃死したエビについては病理学的及び

ＰＣＲ（Polymerase chain reaction）法による検査を行ってＰＲＤＶによる斃死であることを確認した。

試験結果：本発明の低分子量ＬＰＳ区、高分子量ＬＰＳ区及びＬＰＳ無添加区のＰＲＤＶ感染後におけるクルマエビの累積斃死尾数と斃死率を表５～６に示した。

【表５】

試験区分	感染後の経過日数				
	１	２	３	４	５
１区低分子量 ＬＰＳ２０μg／kg	０	０	０	２※	３
２区低分子量 ＬＰＳ４０μg／kg	０	０	３	４	４
３区低分子量 ＬＰＳ１００μg／kg	１	１	３	３	４
４区高分子量 ＬＰＳ１０００μg／kg	１	１	６	６	６
５区ＰＧ ０．２mg／kg	０	０	２	５	５
６区１，３－Ｇ ５０mg／kg	０	３	５	７	１０
７区ＬＰＳ無添加区	２	４	１３	１４	１５

※数字は累積斃死尾数を示す（他も同じ）

【表６】

試験区分	感染後の経過日数					斃死率（％）
	６	７	８	９	１０	
１区低分子量 ＬＰＳ２０μg／kg	３	３	４	４	４	２０※※
２区低分子量 ＬＰＳ４０μg／kg	６	６	６	７	７	３５※※
３区低分子量 ＬＰＳ１００μg／kg	５	６	８	８	８	４０※※
４区高分子量 ＬＰＳ１０００μg／kg	９	９	１０	１１	１１	５５※※
５区ＰＧ ０．２mg／kg	７	８	８	８	１０	５０※※
６区１，３－Ｇ ５０mg／kg	１０	１１	１１	１２	１２	６０※※
７区ＬＰＳ無添加区	１８	１８	１９	２０	２０	１００

※※　７区との間に有意差（Ｐ＜０．０５）

※※※７区との間に有意差（Ｐ＜０．０１）

ＰＲＤＶによる感染後に、ＬＰＳ無添加飼料を与えた対照区のエビは９日以内に１００％が斃死したのに対し、本発明区の斃死率は低分子量ＬＰＳ２０μｇ区が２０％、４０μｇ区が３５％、１００μｇ区が４０％といずれも低く、対照区との間に有意な差がみられた（Ｐ＜０．０１）。一方、高分子量ＬＰＳを１０００μｇ投与したエビの斃死率は５５％、ＰＧを０．２ｍｇ投与したエビの斃死率は５０％、１，３－Ｇを５０ｍｇ投与したエビの斃死率は６０％であり、低分子量ＬＰＳを投与した各区に比べて多数のエビが斃死した。以上の結果から、本発明の低分子量ＬＰＳはエビのウィルスによる感染を防御し、その効果は従来の高分子量ＬＰＳよりもすぐれていることが明らかである。

実施例６　（魚類の免疫機能に対する活性化作用）

平均体重２３０ｇのブリを２０尾ずつの６群に分け、本発明の１、２、３区には低分子量ＬＰＳをブリの体重１ｋｇ当たり１日量として、それぞれ２０、４０、１００μｇを、また高分子量ＬＰＳを４区には１００μｇ、５区には１０００μｇとなるようにモイストペレットに混合して７日間投与した。６区にはＬＰＳを含まないモイストペレットを与えた。投与開始０、１、５、７日後に５尾ずつのブリから頭腎を摘出し、０．２５％NaCl添加ＲＰＭＩ－１６４０－ＨＡＨ培地を入れたプラスチックシャーレ内で血球細胞を分離し、細胞ろ過器に通して細胞懸濁液を得た。この液をpercoll不連続密度勾配上に重層したのち、１６００ｒｐｍ、２０分間（４℃）の遠心分離を行って白血球層を得た。

この層を採取後、遠心洗浄して１０％ＦＢＳ（Fetal Bovire Serum）を含む０．２５％NaCl添加ＲＰＭＩ－１６４０－Ｈ培地に懸濁し、白血球の細胞数を1×10^{6}細胞／ｍｌに調整した。この白血球懸濁液５００μｌと、ブリ血清でオプソニン化しておいた酵母の懸濁液（1×10^{8}細胞／ｍｌ）５００μｌをシリコン処理したガラス試験管に入れ、１０分おきに撹拌しながら２５℃で６

０分間インキュベートした。インキュベート終了後、ブリ１個体当たり５枚の塗抹標本を作製し、ライト染色を施してオイキットで封入した。さらに、光学顕微鏡によって１標本あたり２００細胞の血球を無作為に観察し、白血球の酵母貪食数を調べ、実施例３と同様の計算式によって貪食指数を求めた。結果を表７～８に示した。

【表７】

試験区分	白血球の貪食指数	
	０	１（日後）
１区低分子量 ＬＰＳ２０μｇ／ｋｇ	７．３±２．３０	１２．７±２．６５　※１
２区低分子量 ＬＰＳ４０μｇ／ｋｇ	７．３±２．３０	１７．９±３．９９　※２
３区低分子量 ＬＰＳ１００μｇ／ｋｇ	７．３±２．３０	１８．６±４．１２　※２
４区高分子量 ＬＰＳ１００μｇ／ｋｇ	７．３±２．３０	６．３±２．２４
５区高分子量 ＬＰＳ１０００μｇ／ｋｇ	７．３±２．３０	８．２±２．１８
６区ＬＰＳ無添加飼料区	７．３±２．３０	６．６±１．１９

※１：６区との間に有意差（Ｐ＜０．０５）

※２：６区との間に有意差（Ｐ＜０．０１）

【表８】

	白血球の貪食指数	
	５	７（日後）
１区低分子量 ＬＰＳ２０μg/kg	３９．２±２．５４　※２	５２．７±４．０８　※２
２区低分子量 ＬＰＳ４０μg/kg	３７．４±４．２８　※２	３７．０±３．１１　※２
３区低分子量 ＬＰＳ１００μg/kg	４２．６±５．３５　※２	３６．５±４．３２　※１
４区低分子量 ＬＰＳ１００μg/kg	１１．２±３．０５	１０．６±２．９６
５区低分子量 ＬＰＳ１０００μg/kg	２２．７±３．１６　※１	３１．８±３．５２　※１
６区ＬＰＳ無添加飼料区	９．０±２．０４	７．７±１．７３

※１：６区との間に有意差（Ｐ＜０．０５）

※２：６区との間に有意差（Ｐ＜０．０１）

表７～８から明らかなように、低分子量ＬＰＳを投与したブリにおける白血球の貪食指数は、いずれの発明区ともに６区に比べて高く、有意な差がみられた（Ｐ＜０．０１、０．０５）。しかし、従来の高分子量ＬＰＳを１００μｇ投与したブリにおける白血球の貪食指数は、投与７日後まで上昇せず、１０００μｇ区においては投与５日後以降に６区よりも有意に高くなった（Ｐ＜０．０１）。以上のことから、本発明の低分子量ＬＰＳは高分子量ＬＰＳよりも極めて微量で、白血球の貪食作用などの魚類の免疫機能を活性化することが明らかである。

実施例７（ブリの腸球菌症に対する予防効果）

平均体重６３ｇのブリを３０尾ずつの５群に分け、本発明の１、２、３区には低分子量ＬＰＳをエビの体重１ｋｇ当り１日量として、それぞれ２０、４０、１００μｇを、また４区には高分子量ＬＰＳを１０００μｇとなるようにモイストペレットに混合して、毎日投与した。５区の対照区には、ＬＰＳを含まないモイストペレットを与えた。投与開始７日後にブリの腸球菌症の原因菌Enterococcus seriolicidaをブリ１尾当たり４．０×１０^{6}細胞となるように腹腔内接種し、接種後１５日間の斃死率を求めた。結果を表９～１０に示した。

【表９】

試験区分	感染後の経過日数								
	1	2	3	4	5	6	7	8	9
1区低分子量 LPS20μg/kg	0	0	0	0	0	0	0	0	1※
2区低分子量 LPS40μg/kg	0	0	0	1	1	2	2	4	4
3区低分子量 LPS100μg/kg	0	0	0	0	0	1	3	3	5
4区高分子量 LPS1000μg/kg	0	0	0	1	1	1	3	3	3
5区LPS無添加区	0	0	1	2	7	7	10	12	16

※　　数字は累積斃死尾数を示す（他も同じ）

【表１０】

試験区分	感染後の経過日数						斃死率（%）
	１０	１１	１２	１３	１４	１５	
1区低分子量 LPS２０μg/kg	3	3	3	3	4	4	１３. ３※※※
2区低分子量 LPS４０μg/kg	7	8	8	8	8	8	２６. ７※※
3区低分子量 LPS１００μg/kg	5	5	5	7	7	7	２３. ３※※
4区高分子量 LPS１０００μg/kg	5	9	10	10	11	11	３６. ７※※
5区LPS無添加区	１６	１６	１７	２２	２２	２２	７３. ３

※※５区との間に有意差（Ｐ＜０．０５）

※※※５区との間に有意差（Ｐ＜０．０１）

Ｅ．Ｓｅｒｉｏｌｉｃｉｄａを接種して１５日後に、ＬＰＳ無添加飼料を与えた対照区のブリの７３．３％が斃死したのに対し、本発明区の斃死率は低分子量ＬＰＳ２０μｇ区が１３．３％、４０μｇ区が２６．７％、１００μｇ区が２３．３％といずれも低く、対照区との間に有意な差が見られた（Ｐ＜０．０５）。一方、高分子量ＬＰＳを１０００μｇ投与したブリの斃死率は３６．７％であり、低分子量ＬＰＳの各区に比べて高い斃死率を示した。以上の結果から、本発明の低分子量ＬＰＳは魚類の細菌による感染を防御し、その効果は従来の高分子ＬＰＳよりもすぐれていることが明らかになった。

産業上の利用可能性

本発明によれば、甲殻類及び魚類の免疫機能を、ごく微量で的確に活性化して感染症を予防し、また、甲殻類及び魚類の斃死を予防し、薬物残留などの公衆衛生上の問題のない、安全な甲殻類及び魚類を育成するための飼料添加剤及び飼料を提供することができる。

請求の範囲

１．グラム陰性の微生物菌体から得られ、タンパク質マーカーを用いてＳＤＳ－ＰＡＧＥ法で測定した分子量が５０００±２０００で、高分子量リポポリサッカライドを実質的に含まない、低分子量リポポリサッカライドを有効成分として含有することを特徴とする免疫賦活、感染症予防効果を有する甲殻類又は魚類用飼料添加剤。

２．請求の範囲第１項の低分子量リポポリサッカライド及び甲殻類又は魚類に許容される担体を含有する甲殻類又は魚類用飼料添加剤。

３．甲殻類又は魚類用飼料添加剤を製造するための請求の範囲第１項の低分子量リポポリサッカライドの使用。

４．請求の範囲第１項の低分子量リポポリサッカライドの有効量を甲殻類又は魚類に投与することを特徴とする甲殻類又は魚類の免疫賦活、感染症予防方法。

５．請求の範囲第１項の低分子量リポポリサッカライドを有効成分として含有することを特徴とする甲殻類又は魚類の斃死予防剤。

６．請求の範囲第１項の低分子量リポポリサッカライド及び薬学的に許容される担体を含有する甲殻類又は魚類の斃死予防剤。

７．甲殻類又は魚類の斃死予防剤を製造するための請求の範囲第１項の低分子量リポポリサッカライドの使用。

８．請求の範囲第１項の低分子量リポポリサッカライドの有効量を甲殻類又は魚類に投与することを特徴とする甲殻類又は魚類の斃死予防方法。

９．グラム陰性の微生物菌体がパントエア属に属する微生物菌体である請求の範囲第１項の甲殻類又は魚類用飼料添加剤。

１０．グラム陰性の微生物菌体がパントエア・アグロメランスである請求

の範囲第９項の甲殻類又は魚類用飼料添加剤。

１１．請求の範囲第１項の飼料添加剤を添加したことを特徴とする甲殻類又は魚類用飼料。

１２．請求の範囲第５項の斃死予防剤を添加したことを特徴とする甲殻類又はび魚類用飼料。

１３．請求の範囲第１１項の飼料を甲殻類又は魚類に与えることを特徴とする甲殻類又は魚類の飼育方法。

１４．請求の範囲第１２項の飼料を甲殻類又は魚類に与えることを特徴とする甲殻類又は魚類の飼育方法。

１５．感染症が甲殻類の急性ウィルス血症、ビブリオ病、寄生症、真菌症、魚類のイリドウィルス感染症、ラブドウィルス病、神経壊死症、伝染性造血器壊死症、類結節症、連鎖球菌症、腸球菌症、ビブリオ病、冷水病、シュードモナス病、滑走細菌症、水カビ病である請求の範囲第１項の甲殻類又は魚類用飼料添加剤。

１６．高分子量リポポリサッカライドが８０００以上の分子量を有するものである請求の範囲第１項の甲殻類又は魚類用飼料添加剤。

EP・US

特許協力条約

PCT

国際調査報告

（法８条、法施行規則第40、41条）
〔ＰＣＴ１８条、ＰＣＴ規則43、44〕

出願人又は代理人の書類記号 ＰＴＨＪ－１０００８	今後の手続きについては、国際調査報告の送付通知様式（ＰＣＴ／ＩＳＡ／２２０）及び下記５を参照すること。	
国際出願番号 ＰＣＴ／ＪＰ００／０１７６４	国際出願日（日.月.年） ２３．０３．００	優先日（日.月.年） ２６．０３．９９
出願人（氏名又は名称） 大鵬薬品工業株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条（ＰＣＴ１８条）の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で ３ ページである。

☐ この調査報告に引用された先行技術文献の写しも添付されている。

１．国際調査報告の基礎
ａ．言語は、下記に示す場合を除くほか、この国際出願がされたものに基づき国際調査を行った。
☐ この国際調査機関に提出された国際出願の翻訳文に基づき国際調査を行った。

ｂ．この国際出願は、ヌクレオチド又はアミノ酸配列を含んでおり、次の配列表に基づき国際調査を行った。
☐ この国際出願に含まれる書面による配列表
☐ この国際出願と共に提出されたフレキシブルディスクによる配列表
☐ 出願後に、この国際調査機関に提出された書面による配列表
☐ 出願後に、この国際調査機関に提出されたフレキシブルディスクによる配列表
☐ 出願後に提出した書面による配列表が出願時における国際出願の開示の範囲を超える事項を含まない旨の陳述書の提出があった。
☐ 書面による配列表に記載した配列とフレキシブルディスクによる配列表に記録した配列が同一である旨の陳述書の提出があった。

２．☐ 請求の範囲の一部の調査ができない（第Ｉ欄参照）。

３．☐ 発明の単一性が欠如している（第II欄参照）。

４．発明の名称は ☒ 出願人が提出したものを承認する。
☐ 次に示すように国際調査機関が作成した。

５．要約は ☒ 出願人が提出したものを承認する。
☐ 第III欄に示されているように、法施行規則第47条（ＰＣＴ規則38.2(b)）の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から１カ月以内にこの国際調査機関に意見を提出することができる。

６．要約書とともに公表される図は、
第 ＿＿＿ 図とする。☐ 出願人が示したとおりである。 ☒ なし
☐ 出願人は図を示さなかった。
☐ 本図は発明の特徴を一層よく表している。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ００／０１７６４

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Ｉｎｔ．Ｃｌ．⁷　Ａ２３Ｋ　１／１６，１／１８，　Ａ６１Ｋ　３１／７３９，３１／００，３７／０４

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Ｉｎｔ．Ｃｌ．⁷　Ａ２３Ｋ　１／１６，１／１８，　Ａ６１Ｋ　３１／７３９，３１／００，３７／０４

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

ＢＩＯＳＩＳ，　ＪＯＩＳ

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
X	WO,9623002,A1(Mizuno D.)1.8月.1996(01.08.96) & JP,8-198902,A	1-16
X Y	JP,94-141849,A(杣源一郎)24.5月.1994(24.05.94)　ファミリーなし	1-8,11-16 9,10
X Y	EP,472467,A3(Soma G.)17.3月.1993(17.03.93) & CA,2049533,A & CA,2049548,A & JP,4-99481,A & JP,6-78756,A & US,5281583,A & JP,6-40973,A & JP,6-90745,A & US,5346891,A & US,5494819,A	1-8,11-16 9,10

☒　Ｃ欄の続きにも文献が列挙されている。　　☐　パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
１６．０５．００	23.05.00

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）		
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	長井　啓子	２Ｂ	９１２３
	電話番号　０３－３５８１－１１０１　内線　３２３６		

C（続き）. 関連すると認められる文献		
引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
A	JP, 8-280332, A(全国農業協同組合連合会)29. 10. 1996(29. 10. 96) ﾌｧﾐﾘｰなし	1-16
A	JP, 10-279486, A(太陽化学株式会社)20. 10. 1998(20. 10. 98) ﾌｧﾐﾘｰなし	1-16
A	EP, 592220, A3(Eisai Co. Ltd.)23. 11. 1994(23. 11. 94) & JP, 6-116157, A & JP, 6-327412, A & JP, 7-41427, A & JP, 6-116158, A & US, 5556624, A & US, 5556625, A & US, 5624671, A & US, 5628998, A	1-16
A	高橋幸則，養殖，第３４巻第１０号，　第１１７－１２１頁（１９９７）	1-16
A	Fulvio Salati et al., Nippon Suisan Gakkaishi, vol. 53(2), p. 201-204 (1987)	1-16
A	Marilyn J. Odean et al., Infection and Immunity, vol. 58(2), p. 427-432 (1990)	1-16
A	L. W. Clem et al., Development and Comparative Immunology, vol. 9, p. 803-809 (1985)	1-16

A

シリーズ 総論 養魚への免疫賦活物質利用の可能性

養殖魚類に対する免疫賦活物質の活用

高橋　幸則

一、はじめに

最近、養殖魚類やエビ類に免疫賦活物質が使用されはじめた。この背景には、養殖魚介類に依然としてイリドウイルス感染症、連鎖球菌症、類結節症、クルマエビの急性ウイルス血症などの感染症が多発して甚大な被害を受けている深刻な状況がある。哺乳類に比べて魚類やエビ類には感染症による被害が大きい理由としては、魚介類がリンパ節を持たない上に免疫機能が不完全なことが挙げられるが、そのような魚介類に免疫賦活物質を与えることに対して、否定的な考えを抱く人も多い。

そこで、本稿では魚介類やエビ類がどのような免疫機能を持ち、その機能に対して種々の免疫賦活物質がいかなる作用を発揮するのかについて述べ、更に効果的な投与法と問題点および今後の課題に関して論じてみたい。

二、魚介類の免疫機構

魚類は抗体を産生する機能を有するが、その抗体の種類はIgMのみであり、ヒトにみられるようなIgG、IgA、IgD、IgEなどの様々な作用を示す抗体を持たないために、それによる感染防御能はヒトに比べるとはるかに弱い。また、エビ・カニ類をはじめとする無脊椎動物は抗体を産生する機能を持たない。

しかし、魚類やエビ類にも**表1、2**に示すような免疫機構が存在する。非特異的免疫機構とは、特定の病原体に対してではなく、不特定多数の病原体に対して生体を守る機構をいう。このうち、リゾチームは魚類の体表粘液、血液、腎臓などに存在し、補体と協力し合って細菌を溶解する酵素である[1)~9)]。補体は粘液や血液の中に存在する酵素の複合体で、抗原（病原体）と抗体との結合物が存在すると活性化される系と、抗体とは無関係に細菌のリポ多糖類や細胞壁成分によって活性化される系とがあり、いずれの系も細菌に対して溶菌作用を示す[10)~11)]。魚類が持つ補体は哺乳類のそれと比べて、後者の系による働きが前者の系よりも大きい。細胞性防御因子には生体内に侵入した病原体を貪食したのち、スーパーオキシドなどで殺菌し消化するマクロファージ、好中球などの白血球と、異物細胞やウイルスに感染した細胞に対して傷害活性を有するナチュラルキラー細胞が含まれる[12)]。特異的免疫機構とは、特定の病原体に対してTリンパ球がこれを排除する細胞性免疫と、Tリンパ球の指示によってB細胞様リンパ球が産生した抗体（IgM）による液性免疫をいう。そして、魚類は哺乳動物に比べて、非特異的な免疫機構が果たす役割が大きい。

一方、エビ類は抗体を産生する機能を持たないが、細胞性と液性の防御因子によって生体を守っている[13)]。細胞性因子には血球とリンパ様器官などの定着性細胞が知られている。このうち、血球については大顆粒球と小顆粒球および無顆粒球の三種類の存在が知られており、大顆粒球と小顆粒球に高い貪食活性が認められる[14)]。血球が貪食できないような大きな病原体や多量の細菌などが侵入した場合には、血球が層状をなして病原体を包囲したり、組織の細胞と協力し合って結節を形成し、周囲の

表1　魚類の免疫機構

魚類の免疫機構	非特異的免疫機構	液性防御因子	リゾチーム・補体・プロテアーゼ・CRP・トランスフェリン・インターフェロン・自然凝集素
		細胞性防御因子	マクロファージ・好中球・その他の白血球・ナチュラルキラー細胞
	特異的免疫機構	細胞性免疫	Tリンパ球
		液性免疫	抗体(IgM)

表2　エビ類の免疫機構

エビ類の免疫機構	細胞性防御因子	血球(貪食・包囲化・ノジュール形成)
		リンパ様器官・その他の定着性細胞
	液性防御因子	フェノールオキシダーゼ前駆体(proPO)活性化系 フェノールオキシダーゼ
		レクチン
		リゾチーム・殺菌素

細胞から病原体を隔離する[15]。リンパ様器官は中腸腺の前方に位置する小さな組織で、侵入した病原体を捕捉して生体を守る重要な器官である[13]。また、液性防御因子としてはフェノール系の物質を酸化する酵素の前駆体（proPO）活性化系、レクチンおよび殺菌素が知られている。proPO活性化系とは、エビ類の血球に存在するproPOが、生体内に侵入した細菌のリポ多糖類やカビのβ―1、3―グルカン（多糖類）などで活性化されたセリンプロテアーゼによってフェノール酸化酵素（PO）となり、エビ生体内のチロシンやドーパなどのフェノール系の物質を酸化して、最終的にメラニンを生成する一連の反応をいい、この連鎖反応の中間生成物であるキノンや最終産物としてのメラニンが病原体を包囲して殺滅すると考えられている[16]～[19]。

三、免疫賦活物質の定義と一般的作用

免疫賦活物質とは、前述のような魚類、エビ類およびその他の動物の免疫機能を活性化して、細菌、ウイルス、カビ類などによる感染症に対して抵抗力を増強する物質をいい、強されることが知られている[36]～[38]。また、クルマエビでは血球の食食作

病原体に対して直接的に作用を及ぼして生体を守る物質は免疫賦活物質とはいわない。例えば、魚類に免疫賦活物質を与えると、リゾチームや補体の活性が高まり、補体C３成分が増加する[20]～[22]。また、マクロファージや好中球の病原体に対する食食活性が高まるだけではなく、これらの食食細胞のスーパーオキシド産生能を促して、より強く殺菌する[23]。更に、ナチュラルキラー細胞を活性化して、異物細胞に対する傷害活性を高めるほか、マクロファージのインターロイキン１産生能を促すなどの作用を有する[24]～[26]。このように、魚類に対する免疫賦活物質の作用は、非特異的免疫機能の活性化が主であるが、魚類の抗体産生能を高めたとの報告もある[27]。

一方、エビ類に免疫賦活物質を与えると、大顆粒球や小顆粒球の食食能および殺菌能が高まるとともに、仲間の血球を遊走させる活性化因子の放出を促す[13]、[28]、[29]。また、前述のproPO系を活性化して、フェノールオキシダーゼ活性を高め、病原体に対する抵抗力を増強する[13]、[30]。

四、免疫賦活物質の種類と作用

魚類やエビ類の免疫機能を活性化が高まる。また、エビ類に投与すると血球の食食活性およびフェノールオキシダーゼ活性が上昇することが

する物質は多種に及ぶが、本章では既報の研究に見られる代表的な物質について述べる。

(1)細菌と細菌由来のペプチドグリカンおよびリポ多糖類

ある種のグラム陽性菌の菌体末が免疫機能を活性化することが知られているが、その作用を発揮する主成分は細胞壁に含まれる多糖類ペプチドグリカンである。しかし、すべてのグラム陽性菌がこのような作用を示すのではなく、特定の菌種と特定の菌株に強い活性が認められる。また、いくつかのグラム陰性菌にも免疫賦活作用が認められるが、その作用を示す主成分はリポ多糖類（脂質と多糖類の複合体）である。

①*Bifidobacterium thermophilum*由来のペプチドグリカン

ビフィズス菌の一種であるグラム陽性の*B. thermophilum*の細胞壁から抽出したペプチドグリカンを魚類に投与すると、マクロファージや好中球などの食食活性とスーパーオキシド産生能が高まるとともに、リゾチーム活性が上昇する[31]。また、エビ類に投与すると顆粒球の食食活性とスーパーオキシド産生能が高まるとともに、フェノールオキシダーゼ活性が上昇する[32]。このような免

④クモノスカビ菌体

クモノスカビを魚類やエビ類に投与すると食食細胞が活性化され、

疫機能を活性化することによって、ニジマスのビブリオ病、ウナギのパラコロ病、ブリの連鎖球菌症、クルマエビのビブリオ病および急性ウイルス血症などの感染症に対して防御効果を発揮することが知られている[31]～[33]。

②*Mycobacterium butyricum*菌体（Freundの完全アジュバント）

グラム陽性菌である*M. butyricum*菌体はFreundの完全アジュバントと呼ばれ、ワクチンと混合することによって免疫応答能を高める目的で使われているが、本菌体を単独で魚類に投与すると、マクロファージの食食能と殺菌能が高まるほか、ナチュラルキラー細胞が活性化する。その結果、魚類の*Aeromonas salmonicida, A. hydrophila, Vibrio ordalii, V. anguillarum*による感染を防御する[24]、[35]、[36]。

③*Clostridium butyricum*菌体（ミヤイリ菌体）

グラム陽性の酪酸菌の一種である*C. butyricum*菌体を魚類に投与すると、腎臓由来の白血球細胞の食食活性およびスーパーオキシド産生能が高まるとともに、ニジマスのビブリオ病、ヒラメのエドワジエラ症および連鎖球菌症に対する抗病性が増

感染症、サケ科魚類のせっそう病、ビブリオ病、細菌性腎臓病およびエビ類のビブリオ病に対する抗病性が

強されることが知られている[36)～38)]。

また、クルマエビでは血球の貪食作用が活性化されるとともにビブリオ病に対する抵抗性が増強される。

④グラム陰性菌およびそれ由来のリポ多糖類（LPS）

グラム陰性菌およびその細胞壁外層に存在するリポ多糖類を魚類やエビ類に投与すると、白血球が増加したり免疫機能が活性化することが知られている。また、*Edwardsiella tarda*のLPSをウナギに注射すると、白血球の貪食活性およびパラコロ病に対する感染防御能が高まることが報告されている[25),39)]。

(2)キノコ類、カビ類およびそれら由来の多糖類

キノコ類やカビ類の抽出物および菌糸が、免疫機能を活性化することが知られているが、その作用を発揮する主成分はβ—1、3グルカンと呼ばれる多糖類である。

①スエヒロタケ菌糸由来のシゾフィラン

スエヒロタケの菌糸にはシゾフィランという多糖類（β—1、3グルカン）が含まれており、この物質を魚類に投与すると、貪食細胞、補体、リゾチームなどの非特異的な免疫機能が活性化されるほか、抗体産生能が高まる。また、エビ類に投与すると血球の貪食活性およびフェノールオキシダーゼ活性が上昇することが知られている。これらの免疫機能を活性化することによって、コイのエロモナス感染症、ブリの連鎖球菌症、クルマエビのビブリオ病および急性ウイルス血症などの感染症に対する抵抗性を高めることが報告されている[20)～22),29)]。

②シイタケ由来のレンチナン

シイタケにはレンチナンという多糖類（β—1、3グルカン）が含まれており、この物質を魚類に投与すると腎臓由来の白血球の貪食活性が高まるとともに、コイのエロモナス感染症およびブリの連鎖球菌症に対する抵抗力を増強することが知られている[20),21)]。

③不完全菌類のスクレロチウム由来のスクレログルカン

不完全菌類の*Sclerotium glucanicu*にはスクレログルカン（β—1、3グルカン）という多糖類が含まれており、この物質を魚類に投与すると貪食細胞および補体活性が高まり、コイの実験的*E. tarda*感染症やブリの連鎖球菌症に対する抗病性が増強されることが報告されている[21),22)]。

④クモノスカビ菌体

クモノスカビを魚類やエビ類に投与すると貪食細胞が活性化され、種々の感染症に対する抵抗性が高まることが知られている。これらカビ類にはβ—1、3グルカンが含まれていることから、免疫機能を活性化する成分は、主としてこの多糖類であると考えられる。

(3)酵母の細胞壁およびそれ由来の多糖類

酵母の細胞壁にはβ—1、3グルカン、β—1、6グルカンおよびマンナンなどの多糖類が含まれているが、特にβ—1、3グルカンの含有量が多い。また、酵母の細胞壁または細胞壁から抽出したβ—1、3グルカンが魚類やエビ類の免疫機能を活性化することが知られている。例えば、パン酵母の細胞壁から抽出したβ—1、3グルカンを魚類に投与するとマクロファージやその他の白血球の貪食能および殺菌能が高まるとともに、リゾチーム、インターロイキン1および補体などの活性が上昇する。また、エビ類に投与すると血球のスーパーオキシドおよび過酸化水素の産生能が高まる。これらの免疫機能が活性化されることによって、アメリカナマズのエドワジエラ感染症、サケ科魚類のせっそう病、ビブリオ病、細菌性腎臓病およびエビ類のビブリオ病に対する抗病性が高まる[23),40)]。また、ビール酵母の細胞壁を投与した場合においてもパン酵母のそれと同様に、免疫機能が活性化することが知られている。

(4)海藻およびそれ由来の多糖類

コンブの一種である*Laminaria hyperborea*から抽出したラミナランというβ—1、3グルカンをサケのマクロファージの培養液中に加えると、細胞の吸水作用およびスーパーオキシド産生能が高まる[41)]。また、ワカメ、アキヨレモク、イワヒゲ、ハナフノリ、フクロフノリ、スサビノリなどの熱抽出物を魚類に投与すると、コイの実験的*E. tarda*感染症やブリの連鎖球菌症に対する抗病性が増強されることが知られている[42)]。

(5)キチンおよびキトサン

カニ類やエビ類などの甲殻類および昆虫類の殻に含まれるキチンは、N—アセチル—D—グルコサミンが無数に連結した多糖類で、このキチンがN—脱アセチル化したものがキトサンである。これらの物質を魚類に投与すると、白血球の貪食能および殺菌能、補体活性、リゾチーム活

性が高まるとともに、コイのエロモナス感染症、アユのビブリオ病およびブリの類結節症などに対する抗病性が増強されることが知られている。

(6)放線菌由来のペプチド

放線菌の一種である*Streptomyces olivaceogriseus*の培養ろ液から抽出されたペプチド（FK－156、FK－565）を魚類に投与すると、マクロファージの貪食能および殺菌能が高まり、ニジマスのせっそう病などに対する抗病性が増強されることが知られている[43), 44)]。

(7)ラクトフェリン

抗菌性のタンパク質として知られるラクトフェリンを魚類に投与すると、白血球の貪食能および殺菌能が高まるとともに、ニジマスのビブリオ病やマダイの白点病に対する抵抗性が増強されることが報告されている[45), 46)]。

(8)レバミゾール

チアゾール化合物であるレバミゾールは駆虫剤として広く用いられている。この物質を魚類に投与すると、白血球の貪食能、殺菌能および補体活性が高まるとともに、ニジマスのビブリオ病に対する抗病性が増強されることが報告されている[47), 48)]。

五、使用上の留意点および適正使用法

免疫賦活物質は、投与量および投与方法が適切でないと十分な効果が発揮されない。抗生物質であれば、耐性菌による感染症でない限り最少有効量以上の量を与えれば治療効果が現れるが、免疫賦活物質には有効量に下限と上限があるとともに、連続して投与する場合の期間に限度がある物質が多い。例えば、Aという物質の場合には魚類およびエビ類の体重一kgあたりの一日量として、〇・二mgが適正量であり、この適正量の一〇倍以上の量を与えると、魚の免疫機能が投与しない魚と同じレベルになる。免疫賦活物質のこのような性質は、作用が明確な物質ほど、その傾向が強い。投与期間については、長期間にわたって連続投与してもさしつかえない物質もあるが、間欠的に投与した方が効果が持続的に発揮されるものが多い。例えば前述のA物質の場合には四日連続投与後三日休むか、七日間投与後七日間休む、あるいは一日おきに投与するなどの間欠投与がよく、連続的に投与する場合には二ヵ月間を限度とし、必ず無投与期間をおいて再投与をすると、物質が持つ本来の作用が十分に発揮される。

また、投与時期および期間については、少なくとも病気の多発シーズンは継続して投与することが望ましい。細菌感染症の場合には免疫賦活物質の投与を中止したからといって、数日後に再発することは少ないが、ウイルス病の場合は投与を止めると再発することがある。これは、免疫賦活物質の投与期間中にウイルスが侵入すると、物質の作用で発病は抑えられているものの、生体内ではウイルスが生存しているために、投与の中止とともに発病することがあるからだ。従って、免疫賦活物質の継続期間は、被害の大きな主要疾病の発生し始める時期から終息する時期まで、すなわちイリドウイルス感染症やクルマエビの急性ウイルス血症などでは、水温が一七～一八℃に下降するまで投与することが望ましい。

六、現状における問題点と今後の課題

私達の研究室では、これまでに数多くの免疫賦活物質について、その有用性を評価してきた。研究対象とした物質のうち、数種の物質については著しい免疫活性化作用が認められたにも係わらず、養殖現場で用いられているそれらの物質の評価は、有効である場合と効果が明確に現れない場合とに分かれる。実験室で効果が認められた物質が、なぜ現場では無効なのであろうか。この原因としては、免疫賦活物質が動物の本来的な免疫機能を活性化して、感染症に対する防御効果を間接的に発揮する性質のものであって、抗生物質のように病原菌を直接的に死滅させて治療効果を発揮する物質ではないことが挙げられる。すなわち、魚類やエビ類の免疫機能が活性化しないような条件下（例えば低酸素の環境、過密、ビタミン欠乏飼料の給与やクルマエビでは特に酸化還元電位の低いヘドロ化した底質環境）などで飼育されている場合には、免疫賦活物質を投与しても十分な効果が得られない。このように、免疫賦活物質の使用現状における最大の問題点は、魚類やエビ類の免疫機能が活性化されないような状況の中で用いられていることである。従って、養殖魚類やエビ類に免疫賦活物質を投与する場合には、飼育管理上の基本的対策を講じた上で用いることが今後の課題であろう。

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ORIGINAL ARTICLE

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Intradermal administration of lipopolysaccharide in treatment of human cancer

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Abstract Lipopolysaccharide (LPS) has been recognized as a potent antitumor agent in animal tumor models; however, its use in human cancer therapy has been limited to only one trial, in which LPS from *Salmonella* was given intravenously. It was not very successful because of poor tumor response and was also toxic. We originally developed LPS prepared from *Pantoea agglomerans* (LPSp), and this was a well-purified, small-molecular-mass (5 kDa) agent. We chose intradermal rather than intravenous administration in the hope that the former would release LPS slowly into the bloodstream, and thus be less toxic while preserving antitumor activity. In our animal tumor models, intradermal administration was indeed less toxic and more beneficial for tumor regression than intravenous administration. We made a pilot study with intradermal administration of LPSp on the treatment of ten advanced cancer patients. Five of them had evaluable tumor, which had failed earlier to respond to conventional chemotherapy. Cyclophosphamide was also administered in this trial, in anticipation of its synergistic effect with LPSp. In this study LPSp was injected intradermally into each patient twice a week, starting with an initial dose of 0.4 ng/kg, and raising it to 600 or 1800 ng/kg. A 400-mg/m^2 dose of cyclophosphamide was given intravenously every 2 weeks. After completion of the dose escalation, the treatment was continued for at least 4 months, and it was found that 1800 ng/kg LPSp was well tolerated. A significant level of cytokines was observed in the sera for at least 8 h. These results indicate higher tolerable doses and remarkably more continuous induction of the cytokines than were reported in a previous study by others using intravenous administration. Three of the five evaluable tumors showed a significant response to our combined therapy. Intradermally administered, LPS was less toxic and elicited a tumor response in combination with cyclophosphamide; it can thus can be applied to cancer treatment even in humans.

Key words Lipopolysaccharide · Tumor necrosis factor · Immunotherapy · Cytokine · LPSp

Introduction

About a century ago Coley (1881–1935) showed the therapeutic efficacy of a mixed bacterial vaccine, so-called Coley's toxin, to human cancer [18]. According to a report by Nauts, the 5-year survival rate without recurrence was 42% in 204 patients with inoperable advanced cancer who received only this vaccine [18]. Regardless of how one interprets this result, this information shows that a patient's immune system, when given appropriate immunostimulation, can attack its own cancer and cause complete regression of the tumor. In that period, however, there was no knowledge of the mechanism involved in tumor regression by Coley's toxin. Unfortunately, clinical interest in Coley's therapy diminished in preference to radiotherapy and chemotherapy in the course of their advent into cancer therapy [18]. It has not developed into an established biotherapy for cancer.

In 1943 Shear and Turner isolated lipopolysaccharide (LPS) as the active agent in Coley's toxin [16], and since that time the mechanism underlying the therapeutic efficacy of LPS for cancer has been studied using animal models. LPS is well recognized as a potent immune stimulator as well as a multicytokine inducer, and is known to have potent antitumor activity through a host-defense mechanism. Practically, LPS has been very clearly demonstrated to cause tumor regression in animal tumor models. It is viewed as promising for cancer immunotherapy even in humans, as previously shown in animals. However, its use in human cancer therapy has been limited to only one trial,

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since LPS is rather difficult to prepare as a pure and stable product and is also toxic. The one trial was performed by Engelhardt et al. using LPS from *Salmonella*, which they injected intravenously into advanced cancer patients [4, 5]. This study showed that the maximum tolerated dose (MTD) was 8.0 ng/kg, and this dose resulted in only 1 case of partial response out of 24 [4]. This indicates that, although LPS can cause tumor regression even in human, its MTD is too low to be effective in human cancer therapy when it is administered by the intravenous route.

LPS prepared from *Pantoea agglomerans* (LPSp) was originally developed in our institute. LPSp is a well-purified, small-molecular-mass (5 kDa) LPS which has been standardized and stabilized as an investigational medical drug [8, 13, 17]. For the treatment of cancer patients, we chose intradermal rather than intravenous administration of LPSp in the hope that the former would release LPS slowly into the bloodstream, and thus be less toxic, and preserve antitumor activity. In our animal tumor models, intradermal administration was indeed less toxic and more beneficial for tumor regression than intravenous administration. We examined the antitumor effect of LPSp on various murine syngeneic tumors. Well-established, poorly immunogenic tumors such as MH134 hepatoma and Lewis lung carcinoma were found to regress completely without regrowth when LPSp was administered by the intradermal route in combination with cyclophosphamide [2]. Administration of LPSp alone or cyclophosphamide alone showed suppression of the tumor growth, but neither caused complete regression [2]. Intravenous administration of LPSp showed less antitumor effect than intradermal administration, and did not cause complete regression, even in combination with cyclophosphamide. the median lethal dose for intradermal administration was observed to be five times higher than that for intravenous administration [9].

In a pilot study on the treatment of advanced cancer patients with intradermal administration of LPSp in combination with cyclophosphamide, we evaluated the toxicity and biological effect, and sought evidence of antitumor activity of this combined therapy. We report here that this treatment induced continuous release of cytokines and caused tumor response with less toxicity.

Materials and methods

Preparation of LPS

Lipopolysaccharide was isolated from *Pantoea agglomerans* by the hot phenol method of Westphal in our institute [13]. LPSp was dissolved in saline and prepared in different concentrations.

Selection of patients

Patients eligible for this study were those with histologically confirmed advanced cancer on whom conventional therapies were not expected to have an effect, with 0–3 points of performance status and adequate baseline physiological function, including hematological status, renal function and hepatic function. The exclusion criteria included infectious disease or fever, and cardiac or pulmonary failure. All previous conventional therapies had been discontinued for more than 4 weeks. Signed informed consent was obtained from all ten individuals.

Study design

All the patients were hospitalized for observation while they underwent this therapy. A 200-µl sample of LPSp solution was carefully injected intradermally in an upper limb twice a week; the initial dose was 0.4 ng/kg, since this dose had no adverse effect even when administered by the intravenous route, according to Engelhardt et al. [4, 5]. It was planned to raise the dose to 600 ng in the first five patients and then, after evaluation of the MTD, to raise it in the next five patients to 1800 ng/kg. A 400-mg/m^2 dose of cyclophosphamide was given intravenously every 2 weeks, anticipating its synergistic effect with LPSp, as had been observed earlier in a mouse experiment [1, 2]. When the tumor regressed or was stable, the treatment with these doses was continued for at least a further 4 months. Indomethacin (50 mg), which is a cyclooxygenase inhibitor, was given 30 min before LPSp injection. This was shown to be beneficial for reducing the degree of fever, while not suppressing the release of cytokines such as tumor necrosis factor (TNF) [4, 5]. Vital signs were monitored every 30 min until at least 6 h after injection. The patients were evaluated weekly for complete blood count, coagulation profile, liver function and renal function. An electronic cardiogram and chest roentgenogram were performed after completion of the dose escalation.

Measurement of serum cytokines levels

At the completion of the dose escalation to 600 ng/kg, sera were obtained just before and 1, 2, 4 and 8 h after LPSp injection, and stored at −80 °C until analysis. At the completion of the dose escalation to 1800 ng/kg in cases 6–10, the sera were obtained just before and 2 h after injection of LPSp. The level of TNF, interleukin-6 (IL-6) and granulocyte-colony-stimulating factor (G-CSF) was measured by enzyme-linked immunosorbent assay with commercially available kits (Medgenix Diagnostics, Brussels, Belgium; Endogen Inc., Massachusetts, USA; Amersham, Buckinghamshire, England). The sensitivities of the assay for TNF, IL-6 and G-CSF were 5 pg/ml, 4 pg/ml and 10 pg/ml respectively.

Evaluation of tumor response

Tumor responses were evaluated by physical examination, appropriate roentgenic studies and ultrasound, and assay of serum tumor markers if they were available, at appropriate intervals.

Results

Patients studied

Ten patients were studied (Table 1), ranging in age from 22 to 66 years with 51 years as the median. Clinical diagnoses were uterine cervical cancer in three patients, ovarian cancer in six and malignant brain meningioma in one. Prior therapy other than surgery consisted of chemotherapy in six patients, radiotherapy in two, chemotherapy and radiotherapy in two. Five (cases 1, 2, 4, 6 and 9) out of the ten patients had evaluable tumor at the start of treatment, and in all these five, the tumor had been shown to progress during the prior chemotherapy. In four out of these

Table 1 Characteristics of the patients in this study. Ten patients were studied. Prior therapy other than surgery consisted of chemotherapy in six patients, radiotherapy in two, chemotherapy and radiotherapy in two. Five out of the ten patients had evaluable tumor at the start of treatment, and in all these five the tumor had been shown to progress during the prior therapy. *S* surgery, *C* chemotherapy, *R* irradiation

Case	Age (years)	Diagnosis	Histology	Maximum dose (ng/kg)	Prior therapy	Evaluable tumor
1	59	Ovarian cancer	Clear-cell carcinoma	8	S, C	Yes
2	43	Cervical cancer	Adenocarcinoma	600	S, C, R	Yes
3	44	Cervical cancer	Squamous-cell carcinoma	600	S, R	No
4	61	Ovarian cancer	Endometrioid adenocarcinoma	600	S, C	Yes
5	57	Ovarian cancer	Mucinous cystadenocarcinoma	600	S, C	No
6	50	Ovarian cancer	Clear-cell carcinoma	1800	S, C	Yes
7	52	Ovarian cancer	Undifferentiated carcinoma	1800	S, C	No
8	66	Ovarian cancer	Endometrioid adenocarcinoma	1800	S, C	No
9	22	Brain tumor	Malignant meningioma	1800	S, C, R	Yes
10	53	Cervical cancer	Squamous-cell carcinoma	1800	S, R	No

Table 2 Dose and toxicity profile for intradermal administration of lipopolysaccharide from *P. agglomerans* (LPSp) to patients. LPSp was injected intradermally into ten patients, starting with an initial dose of 0.4 ng/kg. It was planned to raise the dose of 600 ng in the first five patients and then, after evaluation of the maximum tolerated dose, to raise it in the next five patients to 1800 ng/kg. Nine of the ten patients tolerated the administration of LPSp at 600 ng/kg or 1800 ng/kg well, and showed minimum side-effects, including fever, fatigue and mild nausea. One patient had a fever even at a dose of 0.4 ng/kg, probably because of inflammatory carcinomatous peritonitis of her advanced ovarian carcinoma, so the dose was raised to no more than 8.0 ng/kg. The numbers of patients are shown in parentheses

	Dose of LPS (ng/kg)																
	0.4 (10)	1.0 (10)	2.0 (10)	4.0 (10)	8.0 (10)	20.0 (9)	40.0 (9)	100.0 (9)	200.0 (9)	400.0 (9)	600.0 (9)	800.0 (5)	1000 (5)	1200 (5)	1400 (5)	1600 (5)	1800 (5)
Fever																	
WHO grade I	1	1	1	1	1	–	–	1	1	2	2	3	3	4	1	1	–
WHO grade II	–	–	–	–	–	–	–	–	–	1	1	–	–	–	3	3	4
WHO grade III	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fatigue	–	–	–	–	–	–	–	1	1	1	2	2	3	3	4	3	4
Nausea	–	–	–	–	–	–	–	–	–	–	–	–	1	1	1	2	3
Hypotension	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dyspnea	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hepatic toxicity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Renal toxicity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

five (cases 1, 2, 4 and 6), prior chemotherapy included cyclophosphamide and cisplatin as a conventional regimen for ovarian and cervical cancer.

Toxicity

Nine of the ten patients tolerated the administration of LPSp at 600 ng/kg or 1800 ng/kg well, and showed minimum side-effects. One patient had a fever even at the dose of 0.4 ng/kg, probably because of inflammatory carcinomatous peritonitis of her advanced ovarian carcinoma, so the dose was raised to no more than 8.0 ng/kg. The incidence of clinically adverse effects is shown in Table 2. Fever and chills was seen in three out of nine patients (33%) at a dose of 600 ng/kg and in four out of five patients (80%) at 1800 ng/kg. The fever, regardless of its WHO grade, could be inhibited by additional administration of 25 mg or 50 mg indomethacin. All fevers returned to normal within 12 h in all patients. Mild fatigue was observed in most of the patients with fever, and three out of five (60%) had mild nausea at 1800 ng/kg. No other toxicities including hepatic, renal toxicity, hypotension or dyspnea were observed.

Cytokine levels in serum

When dose escalation to 600 ng/kg LPS was completed, the serum levels of TNF, IL-6 and G-CSF were determined by enzyme-linked immunosorbent assay (Table 3). Baseline TNF levels were below 15 ng/kg in all patients. In eight out of nine patients, a significant level of TNF was induced, ranging between 200 pg/ml and 2800 pg/ml. The levels in sera peaked 1 h or 2 h after the injection and at 8 h still remained at a significant amount. The average peak level of TNF was 680 pg/ml, and the other cytokines, G-CSF and IL-6, were observed to be significantly induced following

Table 3 Serum level of tumor necrosis factor (*TNF*), interleukin-6 (*IL-6*) and granulocyte-colony-stimulating factor (*G-CSF*) in nine patients after intradermal administration of LPSp. At the completion of the dose escalation to 600 ng/kg, the sera were obtained just before and 1, 2, 4 and 8 h after LPSp injection. At the completion of the dose escalation of 1800 ng/kg in cases 6–10, the sera were obtained just before and 2 h after injection of LPSp. The levels of TNF, IL-6 and G-CSF were measured by enzyme-linked immunosorbent assay. The sensitivities of the assay for TNF, IL-6 and G-CSF are 5 pg/ml, 4 pg/ml and 10 pg/ml respectively. Baseline TNF levels were below 15 ng/kg in all patients. In eight out of nine patients, a significant level of TNF was induced, ranging between 200 pg/ml and 2800 pg/ml. The levels in sera peaked 1 h or 2 h after the injection and at 8 h still remained significant. The other cytokines, G-CSF and IL-6, were observed to be significantly induced, following the release of TNF, and also to remain for at least 8 h. *ND* not detected

Case	Dose ng/kg	TNF					IL-6					G-CSF				
		0 h	1 h	2 h	4 h	8 h	0 h	1 h	2 h	4 h	8 h	0 h	1 h	2 h	4 h	8 h
2	600	15	460	220	64	52	45	29	186	ND	ND	ND	ND	251	557	236
3	600	9	780	460	146	102	40	75	37	20	36	ND	ND	786	438	150
4	600	15	1027	2800	1227	326	32	165	2020	1560	296	ND	ND	801	425	103
5	600	15	28	202	42	32	ND	ND	10	12	6	ND	ND	62	44	46
6	600	13	102	320	80	40	20	22	707	112	102	ND	11	483	126	86
	1800	13	–	720	–	–	22	–	1020	–	–	ND	–	762	–	–
7	600	10	38	210	50	30	ND	ND	100	18	16	ND	ND	56	24	20
	1800	15	–	520	–	–	ND	–	326	–	–	ND	–	216	–	–
8	600	10	17	18	14	10	ND	ND	25	6	8	ND	ND	ND	ND	ND
	1800	10	–	36	–	–	ND	–	28	–	–	ND	–	ND	ND	ND
9	600	12	182	1800	620	180	11	132	2010	425	282	ND	ND	236	52	50
	1800	10	–	1960	–	–	ND	–	2570	–	–	ND	–	326	–	–
10	600	10	325	120	190	102	ND	106	256	244	182	ND	ND	216	78	36
	1800	8	–	318	–	–	ND	–	362	–	–	ND	–	268	–	–

the release of TNF, and also remained for at least 8 h. When 1800 ng/kg LPSp was administered in cases 6–10, the serum level of the cytokines was determined. The cytokines were induced significantly 2 h after injection in four out of five patients, and the level was higher than that observed in the 600-ng/kg administration.

Schedule of LPS administration

After the 4 months of treatment, the 600 ng/kg or 1800 ng/kg LPSp was adminstered to several patients in this study at various intervals. The influence of the interval between administrations was determined by assaying toxicity and

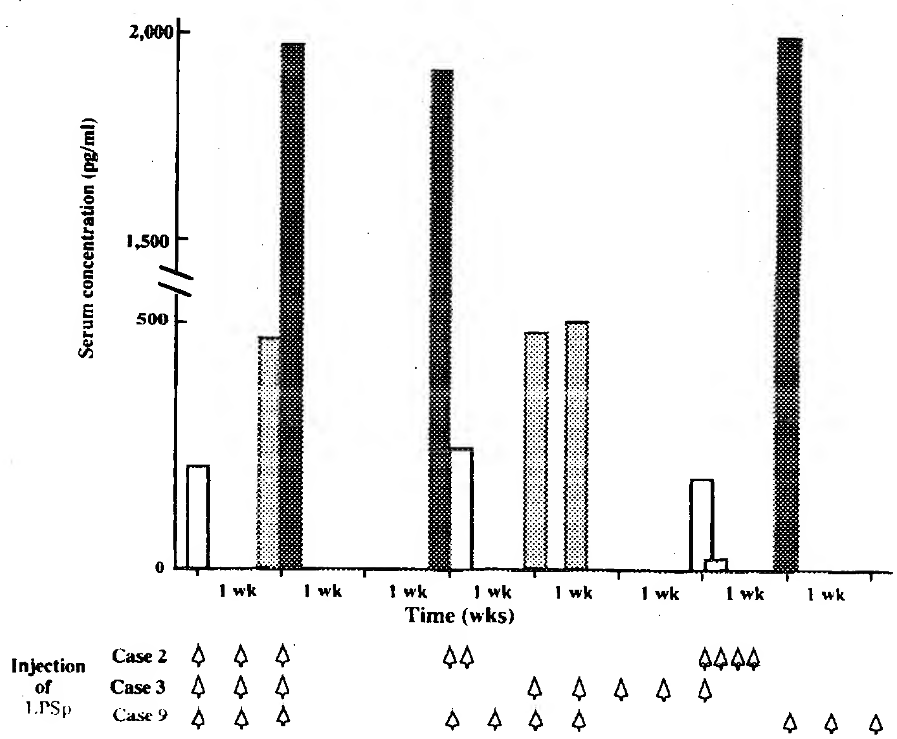

Fig. 1 The serum level of tumor necrosis factor (TNF) in cases 2 (□), 3 (▩) and 9 (□) after intradermal administration of lipopolysaccharide from *P. agglomerans* (*LPSp*) at various intervals. After the 4 months of treatment, the LPSp was administered at various intervals to patients 2, 3 and 9. The time of injection is indicated in each patient. (♤). Serum samples were obtained 2 h after LPSp injectin on several occasions. The serum level of TNF is shown. As in case 2, daily consecutive injections resulted in remarkable attenuation of TNF response. However, there was no difference in induction of TNFα between repeated injections twice a week and those at longer intervals

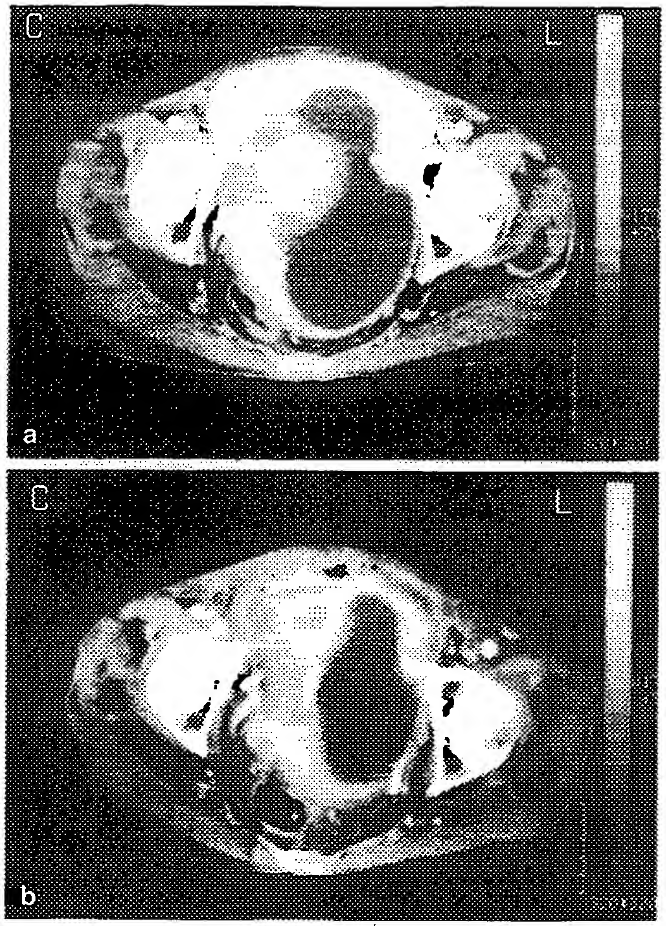

Fig. 2a, b Computed tomography (CT) scan film of the pelvis of patient 4 before (**a**) and after (**b**) treatment. The patient had a giant tumor of ovarian cancer in the pelvis. After treatment the tumor decreased 30% in size (**b**)

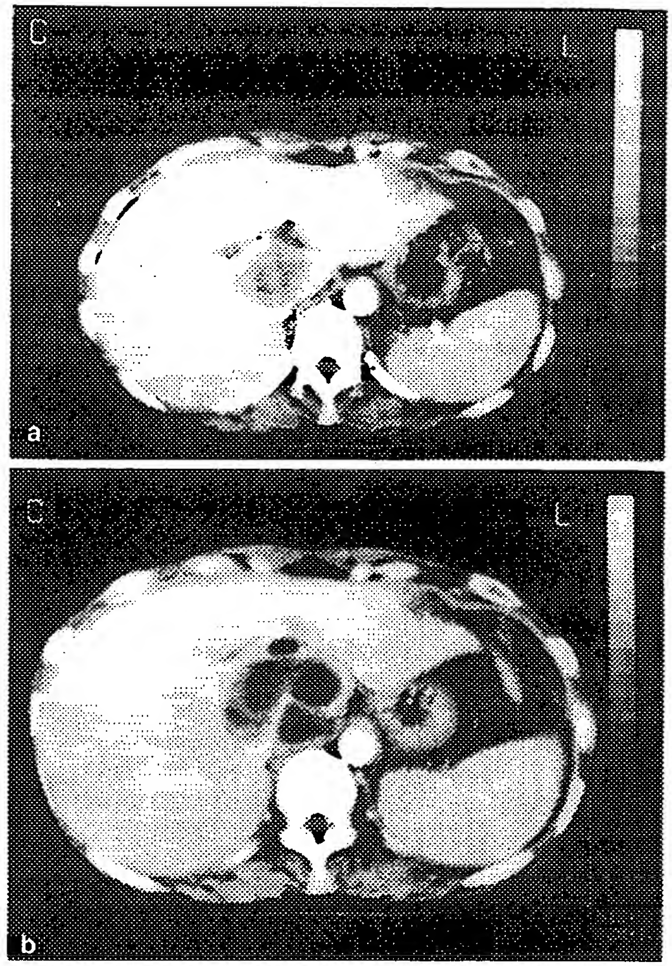

Fig. 3a, b CT scan of the liver of patient 6 before (**a**) and after (**b**) treatment. This patient with ovarian cancer had multiple metastatic tumors in the liver (a). After treatment the density of the tumor remarkably decreased in CT scan (b). The lysis of these tumors was obvious

cytokine release in these patients, but no toxicity higher than that observed during the earlier 4 months of treatment was seen. Serum samples were obtained 2 h after LPSp injection on several occasions, and the level of TNFα was determined (Fig. 1). As shown in case 2, consecutive daily injections resulted in a remarkable attenuation of the TNFα response; however, there was no difference in induction of TNFα between injections repeated twice a week and those at longer intervals.

Tumor response

Five out of ten patients were evaluable for tumor response. In one, the dose was raised to no more than 8.0 ng/kg. In four out of five patients (cases 2, 4, 6 and 9), tumor response could be evaluated after 4 months of treatment with 600 ng/kg or 1800 ng/kg. In three of these four, a favorable change in the tumor was noted. Minor response with significant decrease of tumor markers was seen in two patients (cases 4 and 9), one of whom had a giant tumor of ovarian cancer in the pelvis and the tumor decreased 30% in size (Fig. 2) after the treatment; the other patient had malignant meningioma with extracranial invasion. The invasive tumor at the neck decreased in size, which was judged to be a minor response. In one patient with ovarian cancer with multiple metastatic tumors in the liver (case 6), the lysis of these tumors was clearly demonstrated by computed tomography scan (Fig. 3). In one patient (case 2), the tumor remained stable during the 4 months of treatment.

Discussion

In an earlier mouse experiment, we had found that antitumor activity of intradermally administered LPSp was augmented by repeated injections at shorter intervals and combination with cyclophosphamide [2]. As a feasible

schedule of clinical application, expecting a more favorable therapeutic effect, we selected frequently repeated administration of LPSp, i.e. twice a week. In such a schedule of repeated injection, however, development of LPS tolerance must be taken into consideration. Previous reports have shown that this was greatly influenced by several factors. It was enhanced by application of larger amounts of LPS, shorter intervals between the injections and a higher content of protein in the agent [5, 7]. One earlier report showed that during intravenous administration of 4.0 ng/kg LPS to humans, monitored by release of TNFα, tolerance was developed with repeated weekly injections, but was less with bi-weekly injections [5]. Another report showed that repeated injections of 30 ng of LPS every 2 days did not lead to tolerance to induction of leukocytosis of LPS, but to tolerance to pyrogenic properties [7]. In our administration schedule, using intradermal injection of less than the MTD of LPSp, it was difficult to predict to what degree tolerance was developed. In this pilot study, the development of tolerance was not evaluated in detail. However, we have some results that suggest that tolerance of LPSp was not greatly developed in this study. To several patients of our study, after the 4-month treatment, the LPSp was administered at intervals of more than 2 weeks. However, we observed that there was not much increase of toxicity or release of cytokines even at these intervals. Further controlled study is necessary to determine what dosage and intervals result in tolerance of LPSp by intradermal administration.

This study demonstrated that, when administered by the intradermal route twice a week, 1800 ng/kg LPSp was much less toxic than when given intravenously and this dose was apparently still below the MTD. By the intravenous route, the MTD of LPS administered repeatedly at weekly intervals was reported to be 8.0 ng/kg, and that of first challenge was determined to be 4.0 ng/kg [4]. The MTD in this trial is much higher than that for intravenous injection. The bacterial origin of LPS used in our study was different from that used by Engelhardt et al., which might have allowed a higher MTD for the LPSp. The biological activities of LPSp were extensively studied in our institute using various animal models [8, 13], and compared to those of LPS of other bacterial origins. These studies revealed that, when given intravenously, there was not much difference in toxicities between LPSp and the other LPS of higher molecular mass prepared from *Escherichia coli*. It follows that the much higher MTD in our study cannot well be explained by the difference in bacterial origin; it might instead be due to the route of administration, apart from the bacterial origin or administration schedule.

The cytokines were significantly released after intradermal administration of 600 ng/kg LPSp. Earlier studies have shown that administration of LPS induces release of cytokines such as TNF and IL-6 [4, 5, 6, 12]. Michie et al. measured serum TNF levels in 13 healthy volunteers after intravenous administration of LPS from *E. coli* [12], and showed that 4.0 ng/kg elicited about 240 pg/ml TNF at the peak level. Engelhardt et al. reported that the peak level of TNF in sera was roughly 9000 pg/ml or 1500 pg/ml when the MTD or 1.0–2.0 ng/kg LPS respectively was administered [5]. In these studies the concentration of these cytokines returned to pretreatment levels within only 3–4 h after injection [4, 5, 12]. Our results showed that the peak level of cytokines was lower, but was much more continuous. The peak level of TNF after 600 ng/kg LPSp injection ranged between 200 pg/ml and 2800 pg/ml and a significant level of TNF was maintained for 8 h or more. These doses of LPSp might induce tumor regression and be close to optimal biological doses, judging from the results that the peak TNF level that appeared in sera following intradermal administration of LPSp was about 5000 pg/ml in mice showing complete tumor regression (unpublished observations). Intradermally administered LPSp was absorbed very slowly and stayed in the dermis at the site of injection for more than 48 h [9]. It may thus have a priming and triggering effect on dendritic cells and Langerhans cells in the dermis, and these activated cells and LPSp may continuously enter the bloodstream and reach the local tumor site, resulting in the continuous release of TNF [9, 17]. These effects might be the cause of the continuous release of the serum cytokines observed in this study. In the mouse experiment we evaluated TNF induction following intradermal administration of LPSp, and found that TNF was continuously induced in the tumor site as well as in sera. A significant amount of TNF was still present in the tumor site even 24 h after intradermal administration (unpublished observation). The patients showed marked individual differences of cytokine release in response to LPSp. There seemed to be a tendency for a greater amount of cytokines to be induced by LPSp in patients with a large evaluable tumor. No clear relation was observed between the amount of cytokines induced and the tumor response obtained, since the number of patients in this study was low. In our trial TNF, IL-6 and G-CSF were determined as cytokines released in the sera, since they could be measured using the serum samples. Other members of the cytokine network, such as interferon γ (IFNγ) and IL-12, might be expected to be induced as well.

Several previous studies have demonstrated that induction of endogenous TNF caused tumor regression even in human patients. We developed an anticancer therapy by inducing endogenous TNF with administration of interferon γ and OK432 (a *Streptococcus* preparation) [10]. Although still limited, in 1985 (when we reported it) some efficacy was seen in cancer patients [10]. In the present study with intradermal administration of LPSp combined with cyclophosphamide, we obtained tumor responses in three out of five patients with less toxic doses. We chose to treat the patients with the combination of LPSp and cyclophosphamide rather than LPSp therapy alone since cyclophosphamide was observed in our animal experiments to augment the effect of LPSp synergistically [2]. Several other investigators have also reported that the antitumor effect of endogenous TNF induced by LPS was enhanced by combination with cyclophosphamide [1, 14]. They showed that its ability to enhance the antitumor effect was related to the elimination of some of the immunosuppressive mechanisms that negatively regulated LPS-induced effects. The five

patients had failed to respond to prior chemotherapy, suggesting that the tumor response was not due to cyclophosphamide alone, but to its combination with LPSp and CPM. Continuous release of cytokines, but at a lower peak level, might be beneficial in achieving an antitumor effect with less toxicity. We report here the results from only 4 months of treatment; in some patients, the treatment was continued, and the evaluation of toxicity and outcome of these long-term treatments is now in progress.

LPS has recently been shown to be a potent stimulator of macrophages, releasing not only TNF but also IL-12 [3]. IL-12 has been noted for its central role in causing cell-mediated immunity [11]. TNF was reported to cause acute hemorrhagic necrosis of the tumor and to be a co-stimulator of IL-12 by accelerating IFNγ production from natural killer cells and T cells [20]. Recombinant products of TNF, IFNγ and IL-12 are now available and clinical application of these cytokines has been attempted. It was found, however, that the efficacy of TNF or IFNγ, when administered exogenously as single agents, was limited by severe toxicity [15, 19]; the efficacy of IL-12 is not yet known as clinical trials are now in progress. Tumor cell killing is mediated by the cooperative action of various immunological cells and cytokines. From this point of view, LPS therapy may be more beneficial for tumor eradication in vivo, than a single-cytokine therapy.

We have found that LPS, when intradermally administered, is less toxic, elicits a tumor response in combination with cyclophosphamide and thus can be applied to cancer treatment even in humans. Our emphasis here is thus on the intradermal route for LPS administration. Further escalation of the doses and combined use with other biological response modifiers may offer even more hope.

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